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Resolving The Nutritional Requirements Of The Bile Acid-Dehydroxylating Gut Bacterium Clostridium Scindens

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Resolving the Nutritional Requirements of the Bile Acid-Dehydroxylating Gut Bacterium *Clostridium scindens*

(TITLE)

BY

Oindrila Paul

THESIS

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Abstract

Compared to the other parts of the human body, the gastrointestinal tract harbors a complex microbiota. The impact of the indigenous microbiota on host physiology is most pronounced in the colon, where the primary bile acids chenodeoxycholic acid and cholic acid are converted, via 7α-dehydroxylation, to the toxic secondary bile acids lithocholic acid and deoxycholic acid, respectively, by *Clostridium scindens*, an obligate anaerobe. Interestingly, other than bile acid dehydroxylation, little is known about the basic physiology of *C. scindens*. Understanding the metabolism of *C. scindens* will hopefully provide much-needed information as to how this gut bacterium impacts human health and disease. The goals of our study were to determine the vitamin and amino acid requirements of *C. scindens* and also to determine if *C. scindens* can convert primary bile acid cholic acid to secondary bile acid deoxycholic acid via 7α-dehydroxylation under defined culture conditions. *C. scindens* VPI 12708 was routinely maintained in anaerobic BHI broth medium at 37°C. When *C. scindens* was transferred from the BHI medium to a defined medium (DM; 25 mM glucose, minerals, metals, bicarbonate, 100% CO_2 gas phase, and cysteine), growth was negligible. Only when DM was supplemented with a vitamin mix (*p*-aminobenzoate, biotin, cyanocobalamin, folate, lipoate, nicotinate, pantothenate, pyridoxal, riboflavin, and thiamine) and an amino acid mix (alanine, arginine, asparagine, aspartate, cystine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine) was growth observed. The leave-one-out technique was subsequently used to determine the specific vitamin(s) and amino acid(s) required for growth. With this technique, two vitamins (riboflavin and pantothenate) and one amino acid (tryptophan) were found to be essential for the growth of *C. scindens*. Growth was also reduced, but not inhibited when pyridoxal or biotin was absent from DM. It was also observed that under defined culture conditions *C. scindens* was able to convert cholic acid to deoxycholic acid via 7α-dehydroxylation while producing two unknown products.

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I wish to express sincere gratitude to my advisor, Dr. Steven Daniel, for his kindness, patience, support, and assistance over the past two years which has led to my personal growth, as well as my growth as a scientist. I would also like to thank the members of my committee Dr. Gary Bulla and Dr. Kai Hung for their assistance with my thesis and also to Dr. Jeffrey Laursen, the Graduate coordinator of Biological Science for his guidance throughout my stay at Eastern.

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I. Introduction

Bile acids, which are a group of water-soluble steroids, are synthesized from cholesterol in the human body. There are two kinds of bile acids: primary and secondary. The primary bile acids are the bile acids that are synthesized directly from cholesterol in the liver. The two primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA). The secondary bile acids are formed from the dehydroxylation of the primary bile acids in the terminal small intestine and large intestine by bacteria. One of the many microorganisms engaged in dehydroxylation is the obligately anaerobic bacterium *Clostridium scindens*. The other bile acid metabolizers include *C. hiranonis*, *C. hylemonae*, *C. leptum*, *C. bifermentans*, and *C. sordelli*. The two secondary bile acids formed from the dehydroxylation of CA and CDCA are deoxycholic acid and lithocholic acid, respectively. Excessive accumulation of secondary bile acids in the intestine can lead to colon cancer and other gastrointestinal diseases [59].

A. Bile Acid Synthesis in Humans

Primary bile acids, namely cholic acid (CA; 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholan-24-oic acid), are the two major human bile acids which are synthesized from cholesterol in a series of reactions in the liver catalyzed by a number of enzymes [66]. Prior to their active secretion into the gallbladder from the liver, they are conjugated to either glycine or taurine [79]. The conjugated bile acids are in a fully ionized state and can be termed as bile salts [79]. After being actively secreted across the canalicular membrane into the gallbladder, the conjugated bile acids are stored in the gallbladder and are subsequently released into the small intestine following intake of food [66, 33]. What causes the secretion of bile is the release of cholecystokinin from the duodenum after a meal which stimulates the gallbladder to contract, resulting bile flow into the duodenum [31]. The bile

acids act as detergents and perform a number of functions like solubilization, digestion, and absorption of cholesterol, fats and fat-soluble vitamins [33]. The bile acids are returned to the liver via the portal blood circulation after being actively absorbed from the intestines in the terminal ileum [33]. This process of circulation of bile acids between the liver, gallbladder, and intestines is referred to as enterohepatic circulation (Fig. 1) and occurs at least 10 times a day [79].

Bile acid transportation across the canalicular membrane of the liver is aided by the bile-salt excretion pump, which consumes ATP for the work, present in the membrane [72]. The conjugation process increases the aqueous solubility of the bile acids making them largely impermeable to the cell membranes of the intestine and duodenum, which in turn makes them unable to leave the intestinal lumen [72]. Thus, by allowing the bile acid levels to rise in the lumen, a sufficient concentration is reached to form micelles, which allow emulsification of lipid and their subsequent absorption [72].

Bile acids can be divided into three categories: free bile acids, glycine conjugated bile acids, and taurine conjugated bile acids [72]. The free bile acids include the primary bile acids which are newly synthesized from cholesterol in the liver, cholic acid (CA) and chenodeoxycholic acid (CDCA), and the secondary bile acids formed in the intestine by microbial metabolism, deoxycholic acid (DCA) and lithocholic acid (LCA). Usually, though not always, the free secondary bile acids are formed in the lower gastrointestinal (GI) tract and are absent in the upper GI [66]. These secondary bile acids constitute ~2% of the total bile and have pKa values from 5.2-6.2 [72]. The glycine conjugated bile acids are the most abundant conjugated bile acids comprising >70% of bile. Conjugation restricts bile acids from entering the epithelial cells so that they remain in the intestinal lumen and do not leak into the intra-cellular spaces causing damage to other organs [72]. The taurine conjugated bile acids represent >20% of the bile and are strong sulphonic acids with detergent properties.

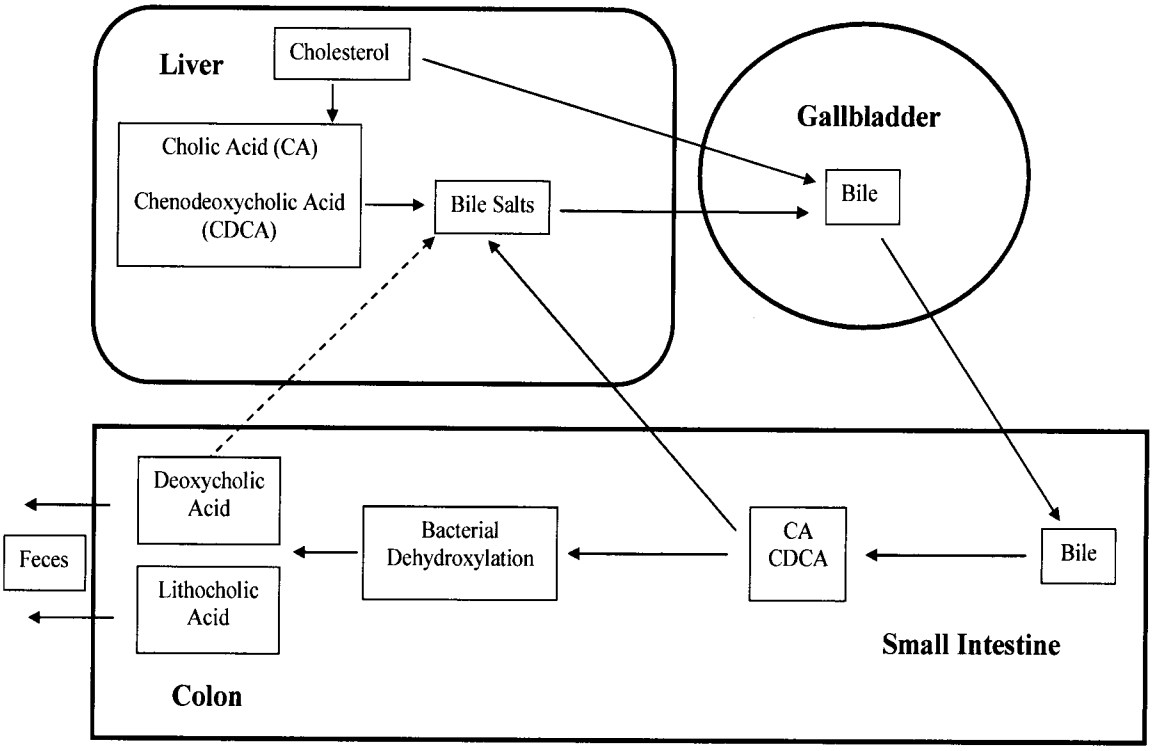


Fig. 1. The enterohepatic circulation of bile acids in humans [79].

Bile acids facilitate the digestion of lipids and their absorption in the small intestine. Dietary cholesterol, phospholipids, bilayers and enzymatic breakdown of triglycerides to fatty acids make up the products of lipid digestion [72]. Being amphipathic, bile acids perform the following two functions: i) emulsification of lipid aggregates and ii) solubilization and transport of lipids in an aqueous environment [51]. As bile acids have detergent like properties, their action on dietary fat causes fat globules to break down or be emulsified into minute droplets. Emulsification aids in digestion by greatly increasing the surface area of fat which makes it available for digestion by lipases as they cannot access the inside of lipid droplets. Bile acids act as lipid carriers and solubilize many lipids by forming micelles which otherwise remain suspended in water. The products of lipid digestion associate with bile acids to form mixed micelles. "The micellar mixture continues down the GI tract to the jejunum, where the contained lipids may diffuse into the epithelium to the portal veins [72]."

B. Microbial Metabolism of Bile Acids in the Human Gut

It was postulated by Louis Pasteur over a century ago that our health was dependent upon our resident microorganisms [51]. The largest density and variety of microbes is found in the human colon. Intestinal pH, bile concentration, and motility are some of the factors responsible for regional differences in the gut microbiota [51]. Bile salts that escape transport in the distal ileum are metabolized by colonic bacteria [66]. "The removal of the 7α-hydroxy substituent on the steroid nucleus is a unique reaction confined to microbial action" [30].

Approximately 600 mg of bile acids are lost into the colon and excreted into the human feces each day due to the poor absorption of bile acids from the small intestine [33]. In the colon, the unabsorbed bile acids are exposed to more than 400 different kinds of bacteria, 99% of which are strict anaerobes [33]. The bacterial species dominating the colon are members of the genera

Bacteroides, Fusobacterium, Eubacterium, and Clostridium [33]. There have been two types of interactions known to exist between intestinal microbes and bile acids: i) the action/s of microbes upon bile acids and ii) the action/s of bile acids upon microbes [52]. When the microbes act on the bile acids, different bile acid biotransformations like deconjugation, oxidation-reduction, and dehydroxylation occur in the gut. All these biotransformations are enzymatic reactions. When excreted bile acids are in the conjugated form, they are mainly conjugated to taurine or glycine [52]. The bond between carbon and nitrogen present in the conjugated bile acids has the ability to withstand cleavage by proteolytic enzymes [52]. Frankel in 1936 was the first to successfully isolate a bacterium having the ability to hydrolyze the conjugated bile acids [21]. Most of the intestinal microbes have enzymes that hydrolyze taurine as well as glycine conjugates [52]. *Enterobacteria* are rarely capable of deconjugation [52]. Deconjugation of primary bile acids like cholic acid and chenodeoxycholic acid are carried out at the C-24 position and is restricted to the large bowel and the terminal ileum when under normal conditions [52]. The microbes responsible for deconjugation include mostly anaerobes like *Bacteroides, Clostridium*, and anaerobic lactobacilli [52]. In cholic acid, the hydroxyl groups are at positions C-3, C-7, C-12 and in chenodeoxycholic acid at C-3 and C-7 [52]. Oxidation occurs at these hydroxyl groups generating various oxo-bile acids [31], e.g. hydroxylation at C-7 yields 7 keto–derivatives [52] (see Figure 2) . The reduction of these oxo-bile acids to the β-position occurs mostly in the liver [33, 52]. Reduction of the oxo-bile acid at C-7 to produce 7β-OH derivatives has not been described for intestinal microbes [52]. Only the strains that contain 3α-OH dehydrogenase are able to form 3β-OH derivatives [52]. The microbes that carry out this process are mainly anaerobes [52]. The enzymes that carry out the oxidation-reduction process are dehydrogenases [52]. The different hydroxyl groups of bile acids are epimerized by the combined action of α- and β-hydroxysteroid dehydrogenases (HSDHs) via an oxo-bile acid intermediate produced by the intestinal microbes [33]. A single species of bacteria with both α- and β-HSDHs can carry out epimerization or by protocooperation between two species of bacteria, one containing α- and the

other containing β-HSDHs, epimerization can also be achieved [33]. When dehydroxylation takes place at the 7α-OH group in the primary bile acids like cholic acid and chenodeoxycholic acid (Figure 2), the reaction leads to the formation of secondary bile acids like deoxycholic acid and lithocholic acid, respectively [31, 52].

C. Diversity of Gut Microorganisms that Transform Bile Acids

"During the enterohepatic circulation, bile salts encounter populations of facultative and anaerobic bacteria of relatively low numbers and diversity in the small intestine [66]." The microbes in the small intestine are mainly responsible for deconjugation and hydroxyl group oxidation in bile salt metabolism [66]. Some of the predominant genera of bacteria inhabiting the small intestinal tract include: *Enterobacteria, Enterococcus, Bacteroides, Clostridium, Lactobacillus and Veillonella* [66]. Even though ileal bile salt absorption is highly efficient, "400-800 mg of bile salts escapes the enterohepatic circulation daily and becomes potential substrates for microbial reactions in the large bowel [66]." *Bacteroides, Eubacterium, Clostridium* are some of the predominant bacterial genera in the large bowel [66]. Even though there are diverse bacterial species present in the intestine, the ability to carry out 7-α dehydroxylation on bile acids is limited to the species of the genus *Clostridium* [80].

Among the many species in the genus *Clostridium*, only six species have been shown to possess bile acid-dehydroxylating activity [39]. These species are *Clostridium scindens, Clostridium hiranonis, Clostridium hylemonae, Clostridium sordelli, Clostridium bifermentans and Clostridium leptum* [39]. It has been found that *C. scindens and C. hiranonis* have 10 times higher 7α-dehydroxylating activity than the other four species [39]. A single bile acid-inducible *(bai)* operon encoding several genes needed for the 7α-dehydroxylating pathway has been detected in *C. scindens and C. hiranonis* [49]. Among the other four species except *C. hylemonae*, they all lack genes of the *bai* operon [39]. Recently in *C. hylemonae* it was

discovered that it even though it is a member of the low bile acid dehydroxylating activity, it consists of a *bai* operon but has the *baiA* gene missing from it [67].

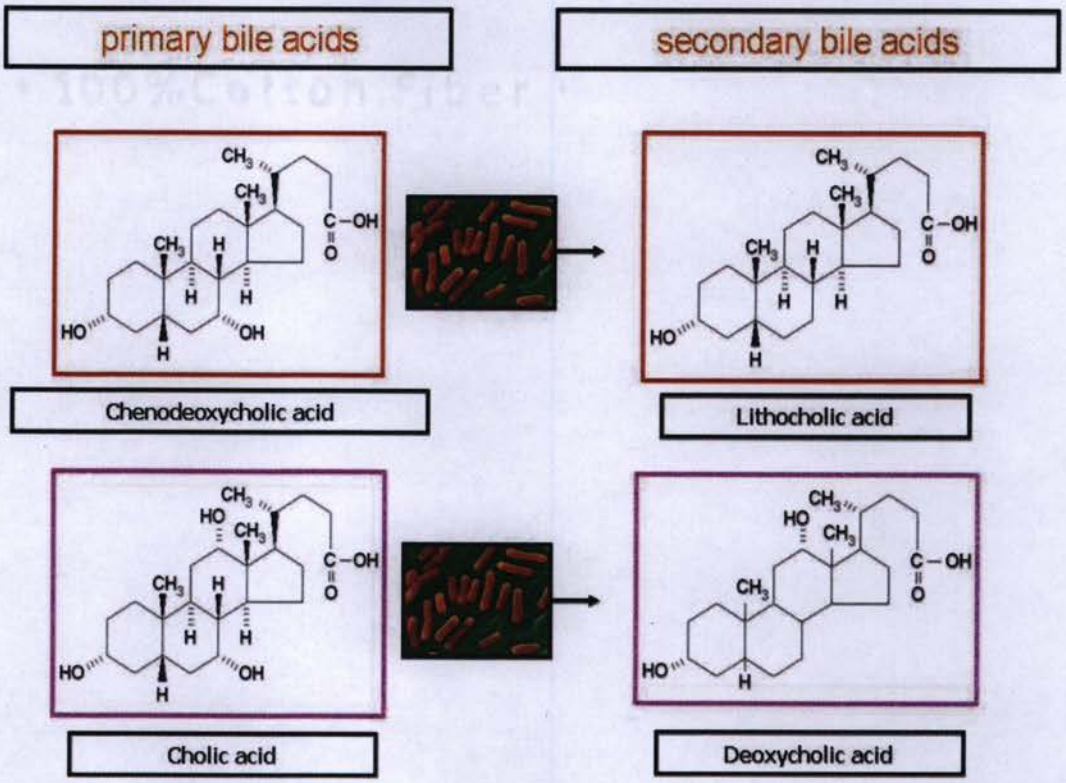


Fig. 2. Bacterial 7α-dehydroxylation of primary bile acids (From Ridlon *et al*, 2006)[66].

D. Enzymology

1. Characteristics of Bile Salt Hydrolases

Deconjugation occurs when the C-24 N-acyl amide bond linking bile acids to their amino acid conjugates are hydrolyzed enzymatically [66]. This process is completed in the large bowel and is a substrate-limiting reaction [66], which means that if the activity of the enzyme is low, the reaction never reaches equilibrium. Even though the substrate level is at a steady level, this level is high and the product thus formed gets rapidly removed by the next step in the pathway never letting the equilibrium to be reached between substrate and product. Bile salt hydrolases (BSH) have been characterized from several species of intestinal bacteria and are part of the choloylglycine hydrolase enzyme family [66]. The bacterial colonization of the lower gastrointestinal tract of higher animals is enhanced by BSH [66]. Many researchers hypothesize that deconjugation is a process of detoxification of bile salts [66]. When a functional BSH is present, there is an accumulation of intracellular free bile acids which become protonated and decrease the H^+-ATPase dependent proton efflux [66]. This means that due to the presence of BSH the bile acids do not conjugate and as a result the proton efflux is lowered. As H^+ is important to carry out the conversion of primary bile acid to toxic secondary bile acid that process is lowered or stopped and hence detoxification of bile acids take place. After the free bile acids are 7α-dehydroxylated by the intestinal bacteria in vivo, "the resultant secondary bile acids tend to precipitate and bind to the insoluble fiber, or they may be absorbed through the colonic membrane, or they may exist in low concentrations in the bacterium's microenvironment [66]." Some bacterial species obtain their cellular carbon, nitrogen and sulfur source from the taurine or glycine released by deconjugation [66].

2. Microbial Bile Acid Hydroxysteroid Dehydrogenase

The 3-, 7-, and 12-hydroxyl groups of bile acids are oxidized and epimerized by hydroxysteroid dehydrogenase (HSDH) present in intestinal bacteria [66]. The reversible change

in stereochemistry from α to β or vice versa with the generation of a stable oxo-bile acid intermediate is termed epimerization of bile acid hydroxyl groups [66]. "Epimerization requires the concerted effort of two position-specific, stereochemically distinct HSDHs of intra-species or interspecies origin [66]." The redox potential of the environment decides the extent of the reversible oxidation and reduction of the bile acid hydroxyl groups by HSDH [66]. More oxo-biles are accumulated when oxygen is added to the culture medium [75]. "HSDHs differ in their reductive and oxidative pH optima, NAD(H), NADP(H) requirements, molecular weight and gene regulation [66]." The reversible, oxidation/reduction between 3-oxo bile acids and 3α- or 3β-hydroxyl bile acids are catalyzed by 3α/β-HSDHs [66]. The 3α-position is favored by the intra-species 3-epimerisation [66]. Epimerization can be carried out in different ways: either by a single species of bacteria which contains both α and β HSDHs or by two different species of bacteria, one having the α-HSDH and the other having the β-HSDH [33].

3. 7α- and 7β-HSDH

The reversible, stereospecific oxidation/reduction of the 7α- and 7β-hydroxyl groups of bile acids are catalyzed by 7α/β-HSDHs [66]. Several *Clostridium* species like *C. scindens* [22], *C. sordelli* [8], *C. absonum* [43], *C. bifermentans* [74] express both 7α and 7β-HSDHs [68]. The 7β HSDH enzymes use NADP(H) as a cofactor [68]. From *C. scindens*, genes encoding 7α-HSDHs have been cloned [5]. It has been seen that 7α/β-HSDH enzymes have a higher affinity for dihydroxy bile acids like CDCA and 7-oxo-LCA than for trihydroxy bile acids like CA and 7-oxo-DCA [66]. Based on sequence similarity, these enzymes appear to belong to the short chain polyol dehydrogenase family [66]. "Regulation of 7α/β-HSDH expression is generally growth phase dependent and inducible by bile acids [66]."

4. 12α- and 12β- HSDH

Some of the members of the genus *Clostridium* have been mainly found to possess 12α/β-HSDH enzymes [66]. *C. leptum* [28], and *C. perfringens* [42] possess 12α-HSDH while *C. tertium*, *C. difficile*, and *C. paraputrificum* possess 12β-HSDH [16]. Like the 7α/β-HSDH enzymes, 12α/β-HSDH have a higher affinity for dihydroxy bile acids like DCA than for trihydroxy bile acids like CA and iso-CA and for free as compared to conjugated bile acids [66]. The addition of 1 mM concentrations of bile acids (DCA > CDCA > CA) to the growth medium represses the activity of 12α-HSDH [66].

5. 7α-Dehydroxylation Pathway

Since secondary bile acids, deoxycholic acid and lithocholic acid, are the most abundant in human feces, among all bile acid biotransformations, 7α-dehydroxylation of primary bile acids carried out by intestinal bacteria in the human colon is the most important [31, 51]. Based on 16S rRNA phylogenetic analysis of the human intestinal microbiota, bacteria capable of this biotransformation are classified in the genus *Clostridium* [12].

After being actively transported into the bacterial cell [49], cholic acid is ligated to coenzyme A (Figure 3) [47]. A 3-oxo-cholyl-CoA conjugate is formed when this cholyl-CoA complex is oxidized by 3α-hydroxysteroid dehydrogenase [48]. The 3-oxo-δ^4-7α,12α dihydroxy bile acid intermediate is further oxidized by a bile acid δ^4-steroid oxidoreductase [33]. The removal of 7α-hydroxy group becomes chemically easier due to the two oxidation steps [33]. A 3-oxo-$\delta^{4,6}$–steroid intermediate is obtained from the dehydration of the 7α-hydroxyl group which is then reduced in three steps yielding deoxycholic acid [33]. It is unclear when CoA is released from the intermediate or how the secondary the bile acid is transported out of the bacterial cell [33].

6. Genetics of Bile Acid 7α-Dehydroxylation

The presence of new polypeptide fragments by SDS-PAGE indicates the induction of the 7α-dehydroxylation activity in the intestinal bacterium *C. scindens* VPI 12708 (also called *Eubacterium* sp. strain VPI 12708). As shown in Figure 4, genes for dehydroxylation are part of a ~12-kb operon which encodes 9 open reading frames and is induced by bile acids [50]. These gene products are associated with bile acid transport or specific steps involved in the 7α-dehydroxylation pathway [11, 23, 6]. Three separate genes encoding 27 kDa Bai proteins have been found in the genome of *Eubacterium* sp. strain VPI 12708. These together are called the *bai*A gene family; *baiA1* and *baiA3* are two identical copies of these genes encoding monocistronic mRNA transcripts [71]. A third gene in the *baiA* family, *baiA2*, is found in the *bai* operon [50]. There is a 92% amino acid sequence identity match by the polypeptides encoded by *baiA2* with polypeptides encoded by *baiA1* and *baiA3* [33]. In addition, there is a considerable DNA sequence identity (69% over 131 bp) shared among the promoter regions of *bai* operon, *baiA1*, and *baiA3* genes and all of them are induced by cholic acid [50, 11, 23, 6, 71].

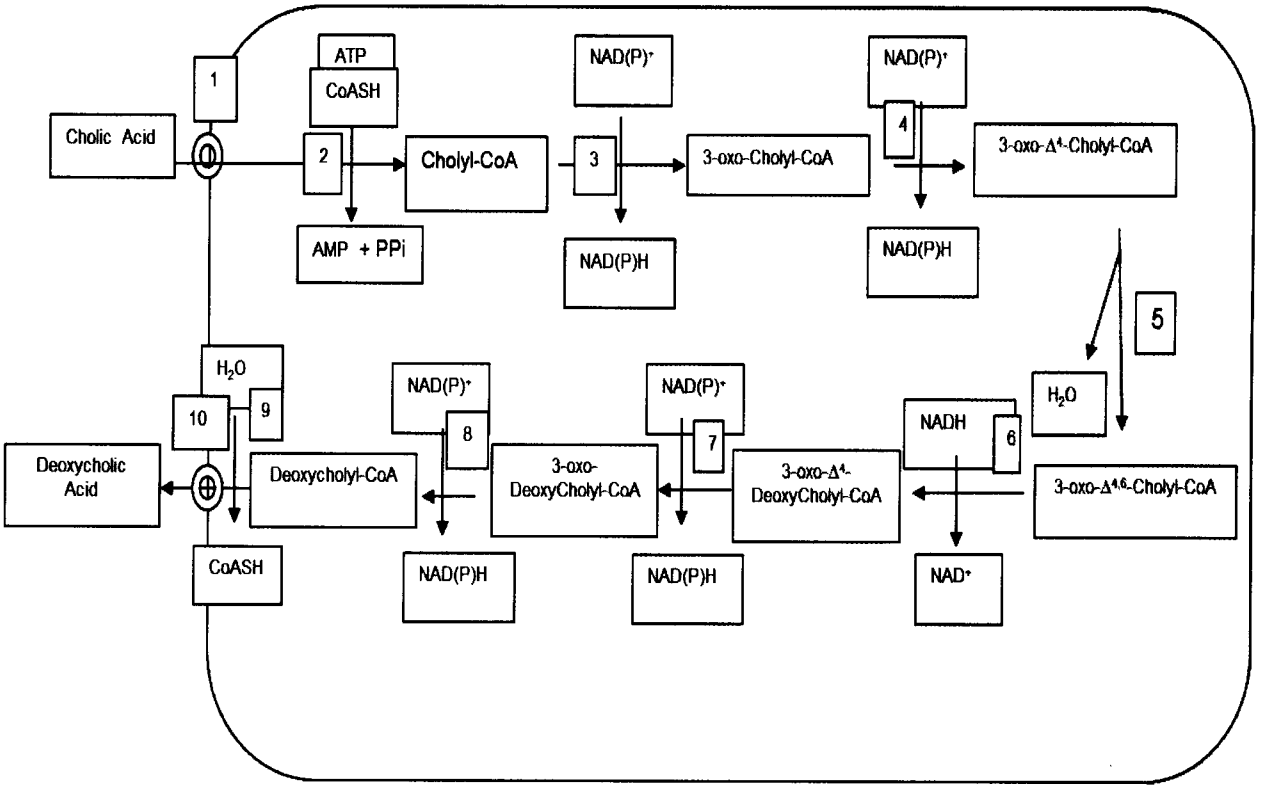


Fig. 3. Proposed pathway for the 7α-dehydroxylation of cholic acid by *C. scindens* (From Baker and Crawford, 1966) [4]. Enzymes and reactions are indicated by numbers: 1, bile acid transporter (uptake); 2, bile acid coenzyme A ligase; 3, 3α-hydroxysteroid dehydrogenase; 4, 3-oxo-Δ^4 oxidoreductase; 5, 7α -dehydratase; 6, Δ^6-oxidoreductase; 7, Δ4-oxidoreductase; 8, 3α-hydroxysteroid oxidoreductase; 9, bile acid coenzyme A hydrolase; 10, bile acid transporter (exporter).

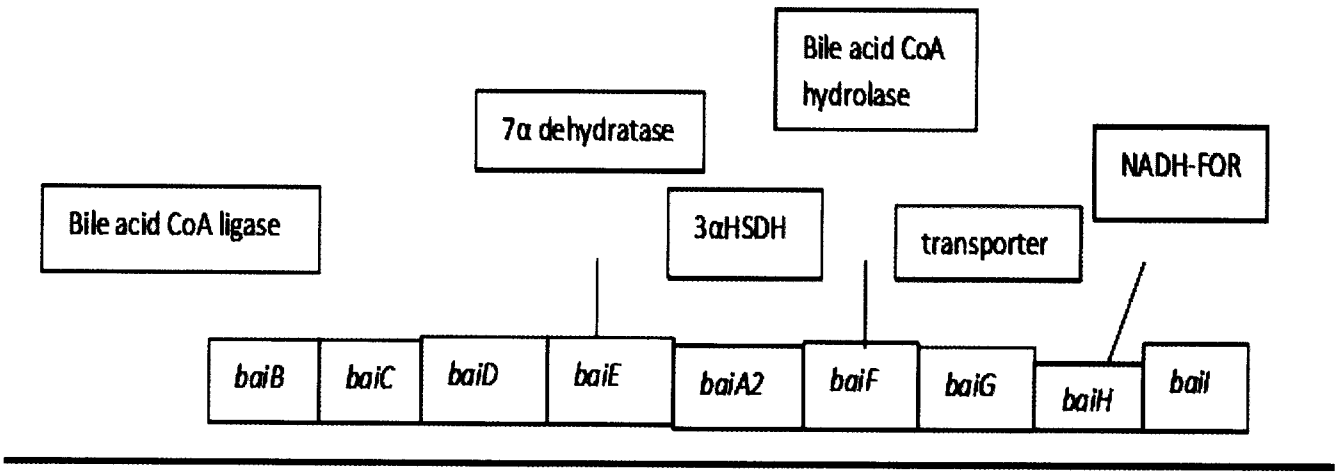


Fig. 4. Bile acid operon in *C. scindens* (3α-HSDH: 3α-hydroxysteroid dehydrogenase; NADH-FOR: NADH-flavin oxidoreductase) [33].

7. Enzymology of Bile Acid 7α-Dehydroxylation

Using polymerase chain reaction, individual genes in the *bai* operon have been amplified and cloned into an IPTG inducible expression vector and expressed in *Escherichia coli* cells [47, 50, 11, 23, 6, 71]. To check if these expressed polypeptides have functions similar to other proteins, amino acid sequence comparisons have been done [33].

a. *baiA* gene. A 27-kDa polypeptide with 249 amino acids is produced by *baiA* genes [33]. There is a significant similarity between the translated amino acid sequence and the short chain alcohol/polyol dehydrogenase family [71] and bacterial bile acid 7-α hydroxysteroid dehydrogenases [5, 8]. Bile acid 3α–hydroxysteroid dehydrogenase (3α-HSDH) activity is observed in the purified gene product when *baiA1* or *baiA2* is expressed in *E. coli* [5]. Although only CoA conjugates of bile acids are recognized by the enzymes encoded by these genes, they can use either NAD^+ or $NADP^+$ as electron acceptors [33]. The physiological significance of multiple genes encoding 3α-HSDH activity is still unknown in the *Eubacterium* species [33]. Between *baiA1* and *baiA2* gene products, no catalytic differences have been observed in vitro [33].

b. *baiB* gene. A 58-kDA polypeptide with 521 amino acids is produced by the *baiB* gene [33]. There is a high degree of similarity between the amino acid sequence and the enzymes that catalyse the ATP-dependent ligation of cyclic carboxylated compounds to AMP or CoA [71]. When *baiB* is expressed in *E. coli*, the *baiB* gene encodes for a bile acid-CoA ligase [33]. For the synthesis of CoA-conjugated bile acids and AMP, the ligation reaction requires unconjugated bile acid, ATP, CoA and Mg^{2+} in vitro [33]. Prior to the oxidation of the 3α-hydroxyl group by the novel 3α-HSDH which is a product of the *baiA* gene family, the conjugation of primary bile acids like cholic acid to CoA is required [33].

c. *baiC* and *baiH* genes. A 72-kDa polypeptide containing 661 amino acids results from the *baiH* gene [33]. This gene has been found to encode for NADH:flavin oxidoreductase activity when expressed in *E. coli* [33]. The amino acid sequence of the *baiH* gene product is similar to the NADH oxidase from *Thermoanaerobium brockii*, triethylamine dehydrogenase from the methylotropic bacterium W3A1, old yellow enzyme from *Saccharomyces carlbergensis*, and the *baiC* gene product from *Eubacterium sp* strain VPI 12708 [23]. The *baiC* gene product is a 59.5 k-Da polypeptide containing 540 amino acids [33]. There is a high degree of similarity between the sequence of the *baiH* and *baiC* genes and their products, suggesting that they are derived from a common ancestral gene [33]. The function of *baiC* gene is unknown.

One mol of FAD, 2 mol of iron and 1 mol of copper per mol of subunit is found in the purified *baiH* gene product, NADH:flavin oxidoreductase [6]. Using NADH as an electron donor, NADH:flavin oxidoreductase reduces quinines, dyes, flavins and molecular oxygen [33]. It is suggested that this enzyme maybe involved in the regulation of the cellular NAD^+/NADH ratio because adding the purified polypeptide to cell extracts of *Eubacterium* VPI 12708 resulted in a change in the ratio of oxidized to reduced bile acid 7α-dehydroxylation intermediates [33].

d. *baiE* and *baiI* genes. A 19.5 k-Da polypeptide with 166 amino acids is the product from the *baiE* gene [11]. The *baiE* gene is associated with a bile acid 7α-dehydratase activity and appears to exist as a dimer in its active form when expressed in *E. coli* [11]. Only 7α, 12α-dihydroxy-3-oxo-4-cholenoic acid and 7α-hydroxy-3-oxo-4-cholenoic acid as substrates are recognized by the 7α-dehydratase encoded by the *baiE* [33]. This enzyme is specific for 7α-dehydration of bile acids since it does not use 7β-hydroxy-3-oxo-4-cholenoic acid [33]. Whether this enzyme will catalyze 7α-dehydration of bile acid-CoA conjugates is unknown [33]. Though there is significant amino acid similarity between the *baiE* gene and *baiI* gene product obtained from the *Eubacterium sp* strain VPI 12708, neither share significant homology with any proteins in the available databases [33]. No activity has been associated with the *baiI* gene [33].

e. *baiF* gene. A 47.5-kDa polypeptide with 426 amino acids is produced by the *baiF* gene [33]. A 60% significant amino acid similarity between the *baiF* gene product and the *caiB* gene product, a 45-kDa carnitine dehydratase of *E.coli*, has been observed [33]. Although the purified *baiF* gene product is involved with the bile acid coenzyme A hydrolase activity [33], no CoA transferase activity or 7α or 7β-dehydratase activity has been detected [8].

f. *baiG* gene. A polypeptide with 477 amino acids is coded by the *baiG* gene and has a significant amino acid sequence homology to membrane transport proteins, including antibiotic resistance transporters [33]. The class K and L TetA proteins from Gram-positive bacteria show the highest degree of similarity [33]. The *baiG* polypeptide has 14 transmembrane domains as seen through computer membrane protein modeling [6] suggesting its function is similar to that of transport proteins. From expression studies in *E. coli*, the *baiG* gene product displays H^+-dependent bile acid transport activity preferring cholic acid and chenodeoxycholic acid [33]. "Secondary degree and conjugated bile acids were transported to much lesser degree [33]."

E. Does 7α/β-Dehydroxylation Benefit the Gut Bacterium?

In the intestinal tract of humans and other animals, the reductive conversion involves the removal of the 7α-hydroxy on the steroid nucleus, also called 7α-dehydroxylation, and converts primary bile acids like cholic acid and deoxycholic acid to secondary bile acids deoxycholic acid and lithocholic acid, respectively [33]. This unique and important reaction is confined to microbial action in the gut [33]. For 7α-dehydroxylating bacteria, the ability to use bile acids as electron acceptors serves as an important niche in the human colon [66]. The multiple oxidative and reductive steps in the 7α/β-dehydroxylation pathway as depicted in Figure 3 result in a net 2-electrons reduction [66]. One of the hypotheses of the energy benefit is that it helps in removal of the secondary bile acids from the environment of the bacteria which is toxic to it thus keeping the

environment toxic free [66]. Another reason could be that the dehydroxylation of primary bile acids provides a selective advantage for the bacteria capable of performing this process as the secondary bile acids are toxic products and these may inhibit or kill bacteria which may be harmful for human body. The bacteria which are sensitive to hydrophobic molecules may be excluded by the production of secondary bile acids [66]. During the dehydroxylation process there is also generation of NAD^+. In glycolysis NAD^+ is reduced to NADH and needs to be oxidized back to NAD^+ for glycolysis to continue. This NAD^+ is provided by fermentation but maybe the NAD^+ generated through dehydroxylation process enters glycolysis and allows continuation of glycolytic pathway.

F. Metabolic Potentials of *Clostridium* Species that Dehydroxylate Bile Acids

The genus *Clostridium* is a group of obligately anaerobic, endospore-forming, Gram-positive rod-shaped bacteria [77]. Six species that are found in this genus, *C. scindens, C. hiranonis, C. hylemonae, C. sordelli, C. bifermentans and C. leptum*, are capable of 7α-dehydroxylation and yield secondary bile acids from primary bile acids [39]. In *C. scindens* and *C. hiranonis*, 7α-dehydroxylating activity levels are ten times higher than those of *C. hylemonae, C. leptum, C. sordelli,* and *C. bifermentans* [39]. Thus, *C. scindens* and *C. hiranonis* have been placed in a "high-activity group" while the remaining organisms fall in a "low-activity group."

In general, *Clostridium* spp. capable of 7α-dehydroxylation are straight to slightly curved, non-encapsulated rods occurring singly or in pairs and are chemoorganotrophs [77]. Colony morphologies are typically less than 2 mm in diameter, circular, convex, and semi-opaque , while their broth cultures are turbid, viscous, and with with stringy or smooth sediment [77]. Most of *Clostridium* spp. grow well when given fermentable carbohydrates like fructose, glucose, galactose, or sucrose [70]. During fermentation, organic acids and gases are often produced [77].

C. scindens got its name from scindens, meaning to split [53]. Endospores are terminal and wider than the vegetative cell [53]. This organism is able to ferment various carbohydrates like D-fructose, D-glucose, lactose, D-mannose, D-ribose, and D-xylose, but not amygdalin, L-arabinose, D-cellobiose, erythritol, esculin, glycogen, inositol, maltose, D-mannitol, D-melezitose, D-melibiose, D-raffinose, L-rhamnose, salicin, D-sorbitol, sucrose, and D-trehalose [53]. Fermentation in peptone-yeast extract glucose broth results in acetic acid and ethanol formation along with abundant production of hydrogen [53]. However, a complete cataloguing of the types and amounts of end products synthesized by *C. scindens* during sugar fermentation has yet to be done. While typical pathways used by *Clostridium* spp. for the fermentation of glucose and other sugars is known (Figure 5), whether *C. scindens* adopts one or more of these fermentation pathways and how each one may be coupled to bile acid dehydroxylation await investigation.

Catabolically, *C. scindens* is incapable of digesting gelatin, milk, and meat [53]. Further, this organisms does not produce indole, lecithinase, lipase, and catalase are not produced, and lactate is not utilized [53]. The organism does not reduce nitrate and is unable to hydrolyze starch [53]. In sulfide-indole-motility medium, hydrogen sulfide production is detected [53]. Thus, the overall metabolic potentials of *C. scindens* appear quite limited. Moreover, this bacterium is normally grown in a complex (undefined) medium containing yeast extract, tryptone, peptone, or brain heart infusion. To date, little, if anything, has been done to address the nutritional requirements (e.g., vitamin and amino acid requirements) of this important gut anaerobe.

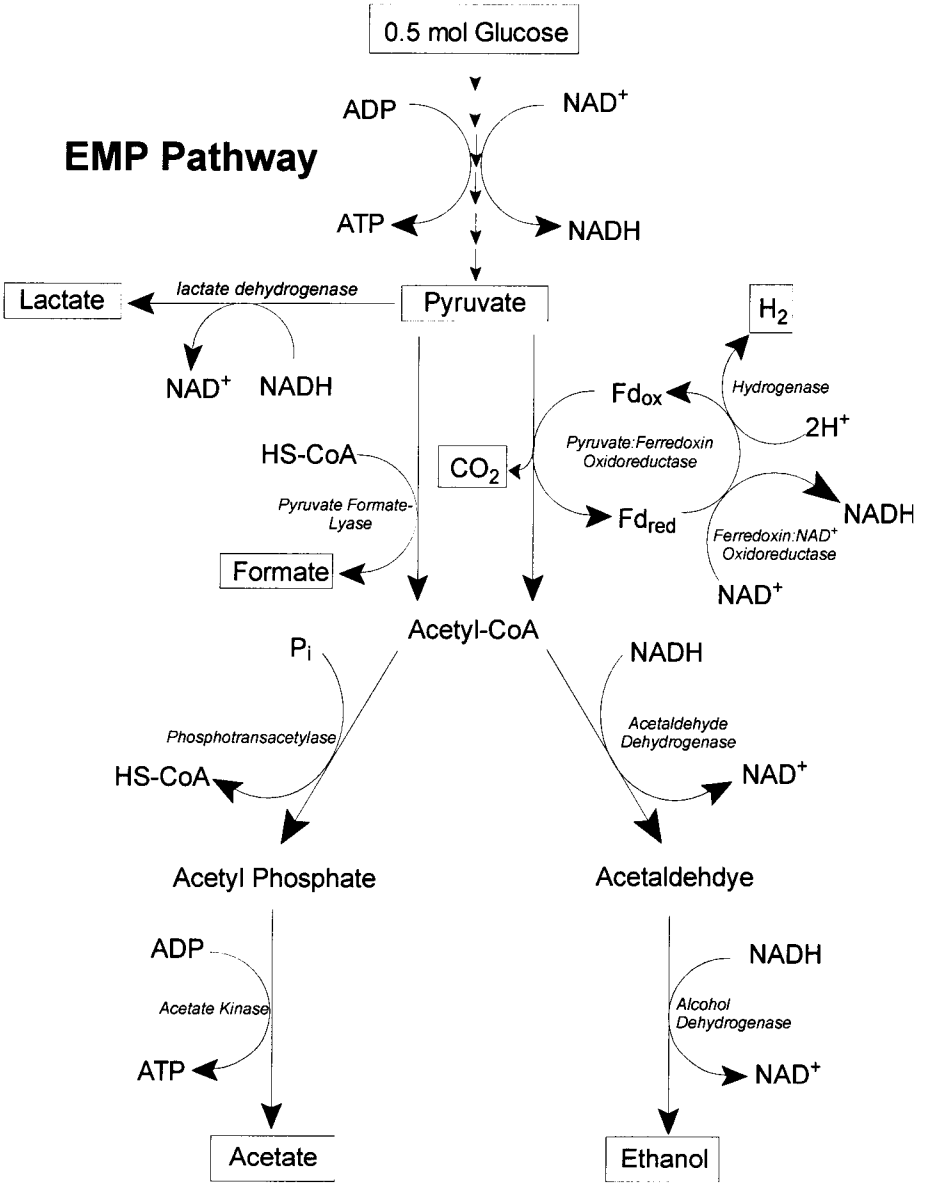


Fig. 5. Typical products formed by *Clostridium* spp. during glucose fermentation [26].

G. Colon Cancer

Colon cancer has been regarded as the third leading cause of cancer deaths in United States (www.cancer.org). According to cancer.org during 2013, colon cancer is expected to be the cause for 50.083 deaths in United States (www.cancer.org). Glycine and taurine conjugate with bile acids and serve as substrates for microbial metabolism [66]. It was stated by Ridlon et al that diet largely affects the extent of taurine conjugation in the bile acid pool [66]. The diet (high in meat) which increases taurine conjugation also increases the chance of colon cancer [66]. Hydrogen sulfide is generated when taurine is metabolized by intestinal bacteria and sulfide generation is linked to increase cell proliferation and thus increase chances of colon cancer [66]. DCA formation in the gut by intestinal 7α-dehydroxylation may also be enhanced by the sulfide production [66]. Secondary bile acids like DCA are considered as "endogenous colon cancer promoters" and are associated with increased rates of colon cancer [59]. Through clinical studies it was seen that patients with colorectal cancer have high accumulations of secondary bile acids in their colonic lumen [59]. Further, studies in animals have also confirmed the potential roles of secondary bile acids in promoting colon cancer [59]. R. Pai *et al* in their studies found that "DCA increases tyrosine phosphorylation and β-catenin levels in colon cancer cells and enhances cell proliferation and cell invasiveness" [59]. This further indicates that "β-catenin is an important molecular target of growth promoting actions of DCA" [59].

II. OBJECTIVES

The objectives of this study were to determine:

(1) If *C. scindens* can convert the primary bile acid cholic acid to the secondary bile acid deoxycholic acid by 7-α dehydroxylation under defined culture conditions.

(2) The vitamin requirements of *C. scindens*.

(3) The amino acid requirements of *C. scindens*.

III. Materials and Methods

Bacterial Strains

Pure cultures of *Clostridium scindens* VPI 12708 were obtained from Dr. Kinchel Doerner, Department of Biological Sciences, Western Kentucky University, Bowling Green, KY and from Dr. Mike Cotta, USDA, ARS, National Center for Agricultural Utilization Research (NCAUR), Peoria, IL. Unless indicated otherwise, all experiments in this project were conducted exclusively with the pure culture of *C. scindens* VPI 12708 obtained from Dr. Doerner.

Preparation of Culture Media

Anaerobic broth culture media used in this study included: Brain Heart Infusion (BHI) broth, undefined medium (UM), and defined medium (DM).

BHI broth. BHI broth was the standard maintenance medium for *C. scindens* VPI 12708 and contained on a per liter basis: 37.0 g Brain Heart Infusion (CM 225; Oxoid Ltd, Cambridge, UK); 5.0 g Bacto™ yeast extract (212750; Becton, Dickinson, and Company, Sparks, MD), 4.0 g glucose (2 g were added to the medium in addition to the 2 g that are normally present in this version of Brain Heart Infusion), and 7.5 g sodium bicarbonate. Culture medium components were added to a 2-l Erlenmeyer flask containing 1 liter of Millipore Reverse Osmosis (RO) water and 1.0 ml of resazurin solution (0.1%; redox indicator). Prior to autoclaving, the pH of the BHI broth was adjusted to 7.0 using 20% NaOH or HCl and an Orion model 230A pH meter (Orion Research Co., Boston, MA) with an Orion semi-micro combination electrode. The BHI medium was prepared anaerobically by boiling the medium under carbon dioxide (CO_2). After boiling, the medium was allowed to cool to room temperature in an ice bath while it was bubbled with CO_2. After the medium cooled, 0.5 g of the reducing agent L-cysteine·HCl·H_2O was added (500

mg per liter). The bubbling of CO_2 was stopped, and the flask headspace was flushed with CO_2 until the color of the medium turned from pink to colorless, indicating the medium was reduced (oxygen free). The medium (10 ml) was dispensed into culture tubes (18 x 150 mm; Bellco series 2048; Bellco Glass Inc., Vineland, NJ) which were being flushed with CO_2. The test tubes were sealed with gray-butyl stoppers and aluminum-crimp seals and then autoclaved at 121°C (15 PSI) for 15 minutes with fast exhaust. The pH of the BHI medium after autoclaving approximated 6.6.

UM. UM was prepared by adding the following components to 950 ml Millipore Reverse Osmosis (RO) water: 4.5 g glucose, 1.0 g Bacto™ yeast extract (212750; Becton, Dickinson, and Company, Sparks, MD), 50.0 ml of mineral solution (10.0 g NaCl, 5.0 g KCl, 10.0 g $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ per 1 liter of mineral solution), 2.0 ml of trace metal solution (1.50 g trisodium nitrilotriacetate, 0.50 g $MnSO_4 \cdot H_2O$, 0.10 g $FeSO_4 \cdot 7H_2O$, 0.10 g $CO(NO_3)_2 \cdot 6H_2O$, 0.10 g $ZnCl_2$, 0.05 g $NiCl_2 \cdot 6H_2O$, 0.05 g H_2SeO_3, 0.010 g $CuSO_4 \cdot 5H_2O$, 0.010 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.010 g H_3BO_3, 0.010 g $Na_2MoO_4 \cdot 2H_2O$, and 0.010 g $Na_2WO_4 \cdot 2H_2O$ per 1 liter of trace metal solution), 1.0 ml of resazurin solution (0.1%), and 7.50 g sodium bicarbonate. Prior to autoclaving, the pH of UM was adjusted to 7.0 using 20% NaOH or HCl and an Orion model 230A pH meter with an Orion semi-micro combination electrode. UM was prepared anaerobically by boiling the medium under carbon dioxide (CO_2). After boiling, the medium was allowed to cool to room temperature in an ice bath while it was bubbled with CO_2. After the medium cooled, 0.5 g of the reducing agent L-cysteine·HCl·H_2O was added (500 mg per liter). The bubbling of CO_2 was stopped, and the flask headspace was flushed with CO_2 until the color of the medium turned from pink to colorless, indicating the medium was reduced (oxygen free). The medium (10 ml) was dispensed into culture tubes (18 x 150 mm; Bellco series 2048; Bellco Glass Inc., Vineland, NJ) which were being flushed with CO_2. The test tubes were sealed with gray-butyl rubber stoppers and aluminum-crimp seals and autoclaved at 121°C (15 PSI) for 15 minutes with fast exhaust. The pH of UM after autoclaving approximated 6.6.

Fig. 6. Components used for making the defined culture medium and BHI.

DM. DM was prepared by adding the following components to 950 ml Millipore Reverse Osmosis (RO) water (see Figure 6 above): 4.5 g glucose, 50.0 ml of mineral solution (10.0 g NaCl, 5.0 g KCl, 10.0 g $(NH_4)_2SO_4$, 5.0 g KH_2PO_4, and 0.5 g $MgSO_4 \cdot 7H_2O$ per 1 liter of mineral solution), 2.0 ml of trace metal solution (1.50 g trisodium nitrilotriacetate, 0.50 g $MnSO_4 \cdot H_2O$, 0.10 g $FeSO_4 \cdot 7H_2O$, 0.10 g $CO(NO_3)_2 \cdot 6H_2O$, 0.10 g $ZnCl_2$, 0.05 g $NiCl_2 \cdot 6H_2O$, 0.05 g H_2SeO_3, 0.010 g $CuSO_4 \cdot 5H_2O$, 0.010 g $AlK(SO_4)_2 \cdot 12H_2O$, 0.010 g H_3BO_3, 0.010 g $Na_2MoO_4 \cdot 2H_2O$, and 0.010 g $Na_2WO_4 \cdot 2H_2O$ per 1 liter of trace metal solution), 1.0 ml of resazurin solution (0.1%), and 7.50 g sodium bicarbonate. Prior to autoclaving, the pH of DM was adjusted to 7.0 using 20% NaOH or HCl and an Orion model 230A pH meter with an Orion semi-micro combination electrode. DM was prepared anaerobically by boiling the medium under carbon dioxide (CO_2). After boiling, the medium was allowed to cool to room temperature in an ice bath while it was

bubbled with CO_2. After the medium cooled, 0.5 g of the reducing agent L-cysteine·HCl·H_2O was added (500 mg per liter). The bubbling of CO_2 was stopped, and the flask headspace was flushed with CO_2 until the medium turned from pink to colorless, indicating it was reduced (oxygen free). The medium (10 ml) was dispensed into culture tubes (18 x 150 mm; Bellco series 2048; Bellco Glass Inc., Vineland, NJ) which were being flushed with CO_2. The tubes were sealed with gray-butyl rubber stoppers and aluminum-crimp seals and autoclaved at 121°C (15 PSI) for 15 minutes with fast exhaust. The pH of DM after autoclaving approximated 6.6.

For nutritional studies, vitamins and amino acids prepared as anoxic (oxygen free), filter-sterilized solutions were aseptically added (0.2 ml of each solution) to culture tubes of sterile DM prior to inoculation as indicated below.

Preparation of Vitamin, Amino Acid, and Bile Acid Stock Solutions

Vitamins. Individual stock solutions were prepared for each of the 10 different vitamins (biotin, thiamine, pantothenic acid, lipoic acid, pyridoxal, *p*-aminobenzoic acid, folic acid, vitamin B_{12}, nicotinic acid, and riboflavin) used in this project. Unless otherwise noted, all chemicals for vitamins, amino acids, and bile acids were purchased from Sigma Aldrich Company, USA. Per individual vitamin solution (100 ml), each contained the following amount of vitamin: 0.01 g biotin, 0.025 g thiamine, 0.025 g pantothenic acid, 0.025 g lipoic acid, 0.01 g pyridoxal, 0.025 g *p*-aminobenzoic acid, 0.010 g folic acid, 0.025 g vitamin B_{12}, 0.025 g nicotinic acid, and 0.025 g riboflavin. The complete vitamin solution (CVS) was prepared by combining 10 ml from each of the individual vitamin solutions (warmed in a 60°C water bath for 15 minutes to help solubilize vitamins) to make 100 ml of CVS (a 1:10 dilution of each vitamin solution). Based on this dilution, the following is the final concentration of each vitamin in the CVS (μg of vitamin per ml of CVS): 10 μg biotin, 25 μg thiamine, 25 μg pantothenic acid, 25 μg lipoic acid, 10 μg

pyridoxal, 25 μg *p*-aminobenzoic acid, 10 μg folic acid, 25 μg vitamin B_{12}, 25 μg nicotinic acid, and 25 μg riboflavin. The individual vitamin solutions and the CVS were stored in the dark at 4°C.

Unless noted otherwise, prior to the addition of CVS to sterile media, non-sterile CVS (10 ml) was sterilized by filtration through sterile 0.2 μm-nylon, 25-mm disposable syringe filters and into empty sterile 18 x 150-mm culture tubes, aseptically sealed with sterile gray-butyl rubber stoppers and aluminum crimp seals and then sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with sterile argon. In some experiments, non-sterile CVS (10 ml) was transferred to 18 x 150-mm culture tubes, sealed with gray-butyl rubber stoppers and aluminum crimp seals, sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with argon, and sterilized by autoclaving at 121°C (15 PSI) for 15 minutes with fast exhaust. Anoxic, sterile solutions of CVS were stored in the dark at room temperature.

Amino acids. Individual stock solutions were prepared for each of the 20 different amino acids used in nutritional studies in this project. Per amino acid solution, each contained the following amount of amino acid per 100 ml of RO water: 4.0 g L-histidine, 4.0 g L-glycine, 4.0 g L-arginine, 4.0 g L-valine, 4.0 g L-threonine, 4.0 g L-isoleucine, 4.0 g L-proline, 4.0 g L-phenylalanine, 4.0 g L-asparagine, 4.0 g L-aspartic acid, 4.0 g L-leucine, 4.0 g L-methionine, 4.0 g L-glutamine, 4.0 g L-glutamic acid, 4.0 g L-serine, 4.0 g L-tryptophan, 4.0 g L-tyrosine, 4.0 g L-alanine, 4.0 g L-lysine, and 4.0 g L-cystine. Some of the amino acids like isoleucine, phenylalanine, asparagine, aspartic acid, leucine, methionine, glutamine, glutamic acid, tryptophan, tyrosine, and cystine would not dissolve easily with water hence a small amount of NaOH was added to solubilize the amino acid. While preparing these amino acid solutions, 2.0 g of each of these amino acids were dissolved in 25 ml water, then 40% NaOH was added in small amounts to these amino acid solutions until they dissolved easily. After adding NaOH, to make up the 50 ml volume, the difference was made up by adding water. The complete amino acid

solution (CAAS) was prepared by combining 5 ml from each of the individual amino acid solutions to make 100 ml of CAAS (a 1:20 dilution of each amino acid solution). Based on this dilution, the final concentration of each amino acid in the CAAS was 2 mg of amino acid per ml of CAAS. The individual amino acid solutions and CAAS were then stored in the dark at 4°C.

Unless noted otherwise, prior to the addition of CAAS to sterile media, non-sterile CAAS (10 ml) was sterilized by filtration through sterile 0.2 μm-nylon, 25-mm disposable syringe filters and into empty sterile 18 x 150-mm culture tubes, aseptically sealed with sterile gray-butyl rubber stoppers and aluminum crimp seals and sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with sterile argon. In some experiments, non-sterile CAAS (10 ml) was transferred to 18 x 150-mm culture tubes, sealed with gray-butyl rubber stoppers and aluminum crimp seals, sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with argon, and sterilized by autoclaving at 121°C (15 PSI) for 15 minutes with fast exhaust. Anoxic, sterile solutions of CAAS were stored in the dark at room temperature.

Bile acids. Individual stock solutions (22 mM) of sodium cholate (M.W. 430.56 g/mol) and sodium deoxycholate (M.W. 414.55 g/mol) were prepared by transferring 0.473 g of sodium cholate and 0.456 g of sodium deoxycholate to 50-ml volumetric flasks and bringing the volume to 50 ml with RO water, respectively. Stock solutions of sodium cholate (CA) and sodium deoxycholate (DCA) were filter sterilized by passing the solutions (10 ml) through sterile 0.2 μm-nylon, 25-mm disposable syringe filters and into empty sterile 18 x 150-mm culture tubes, aseptically sealed with sterile gray-butyl rubber stoppers and aluminum crimp seals, and sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with sterile argon. Anoxic, filter-sterilized solutions were stored in the dark at room temperature.

Vitamin and Amino Acid Supplementation and Inoculation of Culture Media

Anoxic, filter-sterilized solutions of CVS (0.2 ml; 1/54.5 dilution) and/or CAAS (0.2 ml; 1/54.5 dilution) were added to tubes of sterile UM (10 ml) or DM (10 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles and then inoculated (0.5 ml; ~5% inoculum). The final total volume of inoculated culture media was 10.9 ml per tube. Once supplements were added, growth was initiated by anaerobically transferring 0.5 ml of a late-log/stationary phase *C. scindens* VPI 12708 culture using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C.

Following inoculation, the final concentration of vitamins (μg of vitamin per ml of DM) in supplemented DM approximated the following: 0.2 μg biotin, 0.5 μg thiamine, 0.5 μg pantothenic acid, 0.5 μg lipoic acid, 0.2 μg pyridoxal, 0.5 μg *p*-aminobenzoic acid, 0.2 μg folic acid, 0.5 μg vitamin B_{12}, 0.5 μg nicotinic acid, and 0.5 μg riboflavin. The UM contained 0.1% yeast extract, a supplement that contains unknown types and amounts of vitamins; thus, the final concentration of vitamins in UM was unknown.

Following inoculation, the final concentration of each amino acid in supplemented DM approximated 40 mg of amino acid per ml of DM. The UM contained 0.1% yeast extract, a supplement that contains unknown types and amounts of amino acids; thus, the final concentration of amino acids in UM was unknown.

Routine Culture Maintenance and Measuring Growth

C. scindens VPI 12708 was routinely maintained in BHI broth at 37°C. For routine maintenance, as well as for all experiments, growth was initiated by anaerobically transferring 0.5

ml of a late-log/stationary phase *C. scindens* culture using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. In addition to anaerobic BHI broth, some of the other anaerobic culture media used in this study included: UM; UM supplemented with filter-sterilized CVS and CAAS; and DM supplemented with filter-sterilized CVS and CAAS. Cultures were maintained by sequentially transferring them to their respective media after reaching maximum growth. Growth was measured over time as optical density (600 nm) using a Spectronic 20 spectrophotometer (Spectronic Instruments, Inc., Rochester, NY; the optical path width, inner diameter of each culture tube, was 1.5 cm). If culture OD (600 nm) was $\geq$ 0.05 after 3 sequential transfers on a given medium, the culture medium was considered growth supportive for *C. scindens* VPI 12708.

Growth of *C. scindens* VPI 12708 in BHI or DM with 1 mM CA

BHI. An anoxic, filter-sterilized solution of 22 mM CA (0.5 ml; 1/22 dilution) was added to tubes of sterile BHI (10 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles and then inoculated (0.5 ml; ~5% inoculum). The final total volume of inoculated culture media was 11 ml per tube, and, based on the dilution, the final concentration of CA in BHI approximated 1 mM. Growth was initiated by anaerobically transferring 0.5 ml of a late-log/stationary phase BHI culture of *C. scindens* VPI 12708 using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C. Growth was measured over time until the maximum OD was reached, and the culture was then transferred to sterile, CA-supplemented BHI. After 3 sequential passages in BHI + 1 mM CA, growth curves were generated from triplicate tubes of BHI and BHI + 1 mM CA. At the completion of growth, cultures were extracted and used for TLC analyses.

DM. An anoxic, filter-sterilized solution of 22 mM CA (0.5 ml; 1/22.8 dilution) was added to tubes of sterile DM (10 ml) supplemented with anoxic, filter-sterilized CVS (0.2 ml) and CAAS (0.2 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles and then inoculated (0.5 ml; ~5% inoculum). The final total volume of inoculated culture media was 11.4 ml per tube, and, based on the dilution, the final concentration of CA in DM + CVS + CAAS approximated 1 mM. Growth was initiated by anaerobically transferring 0.5 ml of a late-log/stationary phase DM + CVS + CAAS culture of *C. scindens* VPI 12708 using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C. Growth was measured over time until the maximum OD was reached, and the culture was then transferred to sterile, CA-supplemented DM + CVS + CAAS. After 3 sequential passages in DM + CVS + CAAS + 1 mM CA, growth curves were generated from triplicate tubes of inoculated DM + CVS + CAAS and DM + CVS + CAAS + 1 mM CA. At the completion of growth, cultures were extracted and used for TLC analyses.

Thin-Layer Chromatography (TLC) Analyses

Preparation of bile acid standards in methanol. To standardize the TLC method without the need for bile acid extraction from culture media, cholate (CA) and deoxycholate (DCA) standards (1 mM) were prepared directly in methanol. Sodium cholate (0.011 g; M.W. 430.56 g/mol) and sodium deoxycholate (0.010 g; M.W. 414.55 g/mol) were dissolved in 25 ml of methanol each in a 25-ml volumetric flask to give 1 mM CA and 1 mM DCA, respectively.

For preparation of 1 mM CA/DCA (i.e., a mixture containing 1 mM CA and 1 mM DCA), both CA (0.11 g) and DCA (0.010 g) were dissolved in 25 ml of methanol together in a 25-ml volumetric flask. For CA/DCA standards ranging from 0.8 to 0.1 mM, the 1 mM CA/DCA stock solution was further diluted in methanol to achieve the desired concentration: 0.8 mM

CA/DCA standard (800 µl of 1 mM CA/DCA was diluted with 200 µl methanol); 0.6 mM CA/DCA standard (600 µl of 1 mM CA/DCA with 400 µl methanol); 0.4 mM CA/DCA standard (400 µl of 1 mM CA/DCA with 600 µl methanol); 0.2 mM CA/DCA standard (200 µl of 1 mM CA/DCA with 800 µl methanol); and 0.1 mM CA/DCA standard (100 µl of 1 mM CA/DCA with 900 µl methanol). For CA/DCA standards ranging from 0.08 to 0.01 mM, a 0.1 mM CA/DCA stock solution (prepared by mixing 1 ml of the 1 mM CA/DCA stock solution in 9 ml methanol) was further diluted in methanol to achieve the desired concentration: 0.08 mM CA/DCA standard (80 µl of 0.1 mM CA/DCA with 20 µl methanol); 0.06 mM CA/DCA standard (60 µl of 0.1 mM CA/DCA with 40 µl methanol); 0.04 mM CA/DCA standard (40 µl of 0.1 mM CA/DCA with 60 µl methanol); 0.02 mM CA/DCA standard (20 µl of 0.1 mM CA/DCA with 80 µl methanol); and 0.01 mM CA/DCA standard (10 µl of 0.1 mM CA/DCA with 90 µl methanol).

Bile acid standards in BHI. To prepare BHI + 1 mM CA, BHI + 1 mM DCA, and BHI + 1 mM CA/DCA for TLC analyses, BHI was supplemented as shown in Table 1.

Table 1. Bile acid standards in BHI medium for TLC analyses

Standards (mM)	Sterile BHI	Sterile water	Cholate (22 mM)	Deoxycholate (22 mM)	Cholate/ Deoxycholate (22 mM each)
BHI	0.5 ml	0.5 ml			
BHI + Cholate (1)	0.5 ml		0.5 ml		
BHI + Deoxycholate (1)	0.5 ml			0.5 ml	
BHI + Cholate/Deoxycholate (1)	0.5 ml				0.5 ml

Bile acid standards in DM. To prepare DM + 1 mM CA, DM + 1 mM DCA, and DM + 1 mM CA/DCA, sterile DM (containing 0.2 ml of CVS and CAAS added from anoxic, filter-sterilized solutions) was supplemented with bile acids as shown below in Table 2.

Table 2. Bile acid standards in DM + CVS + CAAS for TLC analyses

Standards (mM)	Sterile DM + CVS and CAAS	Sterile water	Cholate (22 mM)	Deoxycholate (22 mM)	Cholate/ Deoxycholate (22 mM)
Sterile DM + CVS + CAAS	0.5 ml	0.5 ml			
Sterile DM + CVS + CAAS + Cholate (1)	0.5 ml		0.5 ml		
Sterile DM + CVS + CAAS + Deoxycholate (1)	0.5 ml			0.5 ml	
Sterile DM + CVS + CAAS + Cholate/Deoxycholate (1)	0.5 ml				0.5 ml

Extraction of standards and cultures. Samples (0.5 ml) from standards prepared in sterile BHI or DM or from BHI or DM cultures of *C. scindens* VPI 12708 were transferred to duplicate labeled 2-ml microcentrifuge tubes. To each sample, 100 µl of 20% HCl was added for acidification in order to convert the bile salts to bile acids. Ethyl acetate (1 ml) was added to each of the duplicate acidified samples. The samples were capped, mixed by vortexing, and spun in a microcentrifuge for 1 minute at 14,000 rpm. The organic phase (top layer) from both samples was transferred to a large-mouth vial, and the combined ethyl acetate fractions were dried at room

temperature under a stream of air. Methanol (100 µl) was added to each vial to resuspend the dried extracts. The resuspended extracts were immediately used for TLC.

Spotting and processing of TLC plates. All manipulations were done a fume hood. Samples (50 µl) of standards or culture extracts were spotted on non-UV, 20 x 20-cm, silica TLC plates with aluminum backing (AL SIL G, 250-µm thick; Whatman, GE Healthcare Life Sciences, Piscataway, NJ) using a 50-µl glass syringe (Figure 7). To minimize spot size, a small amount of sample was applied at a time, allowed to evaporate, and then more sample applied. This was repeated until all of the 50 µl-sample was applied. To speed up the process, a hair dryer (low setting) was used between applications to help evaporation. The syringe was rinsed with methanol between different standards or culture extracts being spotted. The spotted plate was placed in a TLC chamber with 33 ml of solvent (ethyl acetate:cyclohexane:glacial acetic acid) in a 23:7:3 ratio (Figure 7). The solvent was modified when spots became diffused rather than pinpoint. The modified solvent consisted of ethylacetate:chloroform:cyclohexane:glacial acetic acid in 18:5:7:3 ratio. The solvent was allowed to run to almost the top of the plate. The plate was taken out of the chamber, allowed to air dry, and sprayed with a charring agent. The charring agent contained: methanol (150 ml); RO water (150 ml); concentrated H_2SO_4 (10 ml); and $MnCl_2.4H_2O$ (1 g). The plate was charred in an oven at 110°C for 15 minutes. The spots were observed under long UV light and photographed.

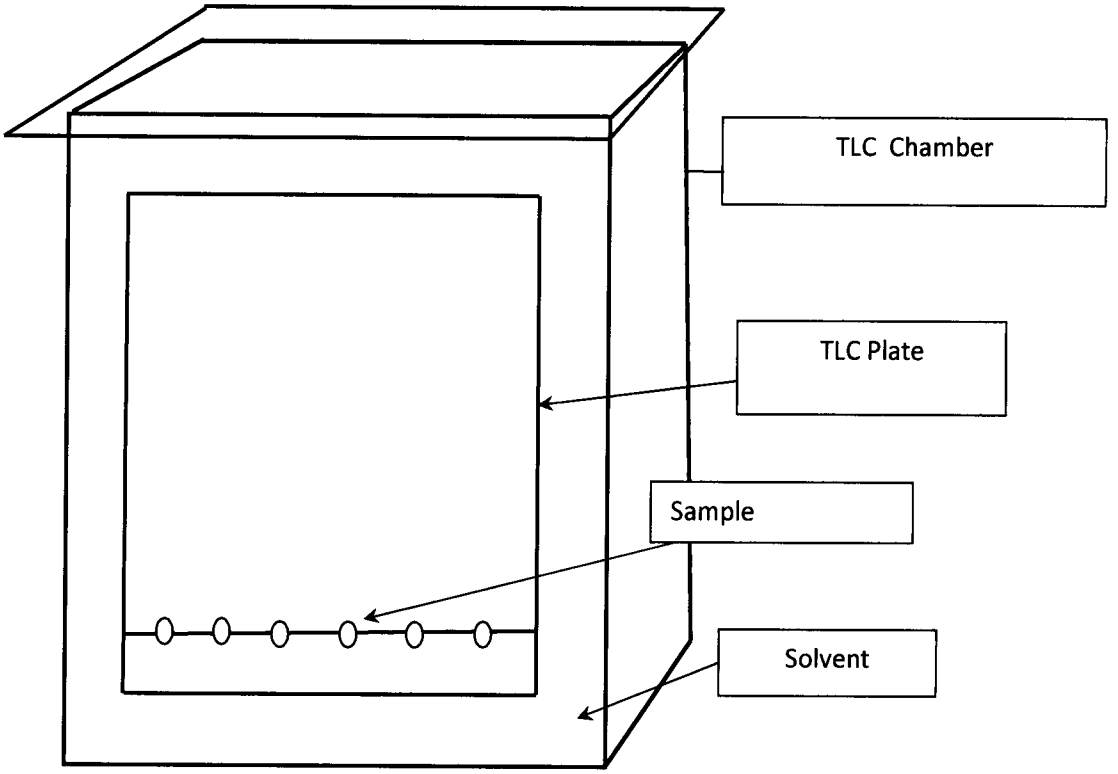


Fig. 7. A typical TLC setup.

Resolving the Vitamin and Amino Acid Requirements of *C. scindens* VPI 12708

Initially, to determine if *C. scindens* VPI 12708 possessed a growth-factor requirement for vitamins and amino acids, growth of the organism was evaluated under the following culture conditions: DM + CVS + CAAS; DM + CVS (no CAAS); DM + CAAS (no CVS); DM (no CVS or CAAS). Anoxic, filter-sterilized solutions of CVS (0.2 ml) and/or CAAS (0.2 ml) were added to tubes of sterile DM (10 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles. If CVS or CAAS was not added, 0.2 ml of anoxic, filter-sterilized RO water was added. If both CVS and CAAS were omitted, 0.4 ml of anoxic, filter-sterilized RO

water was added. Once all supplements were added, growth was initiated by anaerobically transferring 0.5 ml (~5% inoculum) of a late-log/stationary phase DM + CVS + CAAS culture of *C. scindens* VPI 12708 using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C. Growth was measured over time until the maximum OD (600 nm) was reached, and cultures were maintained by sequentially transferring them to their respective media. After 3 sequential passages in a given supplement-deficient medium (DM + CVS; DM + CAAS; and DM), if culture OD (600 nm) was ≤ 0.05, the medium was considered non-growth supportive and hence indicated that a specific growth factor (vitamin or amino acid or both) was required by *C. scindens* VPI 12708 for growth.

Vitamin requirements. For nutritional studies designed to determine the vitamin requirements of *C. scindens* VPI 12708, the "leave-one-out technique" was utilized where 10 different sterile vitamin solutions (100 ml each) were prepared as described above for the CVS, except that each of these solutions was made deficient in a single vitamin by omitting the addition of a vitamin solution. The missing vitamin solution was replaced with 10 ml of RO water. These 10 different single vitamin-deficient solutions (designated as CVS-1), as well as the CVS, were filter sterilized by passing the solutions (10 ml) through sterile 0.2 μm nylon, 25-mm disposable syringe filters and into empty sterile 18 x 150-mm culture tubes, aseptically sealed with sterile gray-butyl stoppers and aluminum crimp seals, and finally sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with sterile argon. Anoxic, filter-sterilized solutions were stored in the dark at room temperature.

Prior to inoculation, anoxic, filter-sterilized solutions of CVS (0.2 ml) and CAAS (0.2 ml) or CAAS (0.2 ml) and CVS-1 (0.2 ml) were added to tubes of sterile DM (10 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles. Once all supplements were added, growth was initiated by anaerobically transferring 0.5 ml (~5%

inoculum) of a late-log/stationary phase DM + CVS + CAAS culture of *C. scindens* VPI 12708 using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C. Growth was measured over time until the maximum OD (600 nm) was reached, and cultures were maintained by sequentially transferring them to their respective media. After 3 sequential passages in a given vitamin-deficient medium (DM + CAAS + CVS-1), if culture OD (600 nm) was ≤ 0.05, the medium was considered non-growth supportive and hence indicated the specific vitamin (the one missing in the CVS-1 solution) that was required by *C. scindens* VPI 12708 for growth.

Amino acid requirements. For nutritional studies designed to determine the amino acid requirements of *C. scindens* VPI 12708, the 20 amino acids were initially divided into 6 different groups since amino acids within a specific group may be interconverted by bacteria. The 6 different groups consisted of the following: glutamine group (L-glutamine, L-glutamate, L-proline, and L-arginine); serine group (L-serine, L-glycine, and L-cystine); aspartate group (L-methionine, L-asparagine, L-aspartate, L-lysine, L-threonine, and L-isoleucine); pyruvate group (L-alanine, L-valine, and L-leucine); aromatic group (L-tryptophan, L-tyrosine, and L-phenylalanine); and histidine group (only L-histidine). Six different amino solutions (100 ml each) were prepared as described above for the CAAS, except that each solution was made deficient in a particular amino acid group by omitting the addition of individual solutions containing the amino acids that were specific to that group. Each individual amino acid solution omitted was replaced with 5 ml of RO water. These 6 different amino acid group-deficient solutions (CAAS-1), as well as the CAAS, were filter sterilized by passing the solutions (10 ml) through sterile 0.2 μm nylon, 25-mm disposable syringe filters and into empty sterile 18 x 150-mm culture tubes, aseptically sealed with sterile gray-butyl stoppers and aluminum crimp seals,

and finally sparged, flushed, and pressurized to 15 PSI (1 bar) over atmospheric pressure with sterile argon. Anoxic, filter-sterilized solutions were stored in the dark at room temperature.

Prior to inoculation, anoxic, filter-sterilized solutions of CVS (0.2 ml) and CAAS (0.2 ml) or CVS (0.2 ml) and CAAS-1 (0.2 ml) were added to tubes of sterile DM (10 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles. Once all supplements were added, growth was initiated by anaerobically transferring 0.5 ml (~5% inoculum) of a late-log/stationary phase DM + CVS + CAAS culture of *C. scindens* VPI 12708 using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C. Growth was measured over time until the maximum OD was reached, and cultures were maintained by sequentially transferring them to their respective media. After 3 sequential passages in a given amino acid group-deficient medium (DM + CVS + CAAS-1), if culture OD (600 nm) was ≤ 0.05, the medium was considered non-growth supportive and hence indicated that one or more amino acids in a specific group were required by *C. scindens* VPI 12708 for growth.

Once the amino group required for growth by *C. scindens* VPI 12708 was identified, the specific amino acid(s) in that group needed for growth was determined by the "leave-one-out technique". The required amino acid group solution was prepared by combining 5 ml of each of the individual amino solutions specific to that group and bringing the volume to 100 ml with RO water. The required group solution was also made deficient in one or more of the amino acids specific to that group by omitting the individual solutions for those amino acids. Each individual amino acid solution omitted was replaced with 5 ml of RO water. All of these solutions were filter sterilized by passing the solutions (10 ml) through sterile 0.2 μm nylon, 25-mm disposable syringe filters and into empty sterile 18 x 150-mm culture tubes, aseptically sealed with sterile gray-butyl stoppers and aluminum crimp seals, and finally sparged, flushed, and pressurized to 15

PSI (1 bar) over atmospheric pressure with sterile argon. Anoxic, filter-sterilized solutions were stored in the dark at room temperature.

Prior to inoculation, anoxic, filter-sterilized solutions of CVS (0.2 ml) and the required amino acid group (0.2 ml) or CVS (0.2 ml) and the required amino acid group deficient in one or more amino acids specific to that group (0.2 ml) were added to tubes of sterile DM (10 ml) using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles. Once supplements were added, growth was initiated by anaerobically transferring 0.5 ml (~5% inoculum) of a late-log/stationary phase DM + CVS + CAAS culture of *C. scindens* VPI 12708 using sterile, degassed 1.0-ml Becton Dickson (BD) syringes and 23-gauge/1-inch BD needles to tubes of sterile culture media. Tubes of inoculated media were incubated vertically in test tube racks in the dark at 37°C. Growth was measured over time until the maximum OD was reached, and cultures were maintained by sequentially transferring them to their respective media. After 3 sequential passages in a given amino acid-deficient medium, if culture OD (600 nm) was ≤ 0.05, the medium was considered non-growth supportive and hence indicated the amino acid in a specific group that was required by *C. scindens* VPI 12708 for growth.

Analysis and Graphing of Data

For nutritional studies, data presented represents the average of duplicate or triplicate measurements unless indicated otherwise. Growth data was analyzed and graphed by Microsoft Excel or by SigmaPlot.

Bioinformatics Tools

The different genes involved in the riboflavin biosynthesis pathway and the pantothenate biosynthesis pathway for *E. coli* K12 strain MG1655 was identified on the Kegg pathway database. Once the gene was identified, its corresponding protein sequence was retrieved from the NCBI protein database under the FASTA format and pasted into the protein blast (blastp) bioinformatics tool. Basic Local Alignment Search Tool (BLAST) is a bioinformatics tool which is used to "find regions of local similarity between sequences" [1].

Once pasted onto the given space for "enter query sequence", the organism against which the BLAST was to run to compare the query sequence was selected from the dropdown box (in this case *Clostridium scindens* ATCC 35704). The genome sequence (draft) of *C. scindens* ATCC 35704 has been determined as part of Human Microbiome Project (HMP) Reference Genomes project and is publicly available at the DOE Joint Genome Institute (http://www.jgi.doe.gov/). After selecting the organism, BLAST was run for comparison analysis. Based on the comparison, a series of "hits" is generated with each hit having a corresponding "E-value". E-value "describes the number of hits one can generate by chance when searching the database of a particular size" [1]. The lower the E-value, the more significant the match is. Negative exponential of E values are considered to be a significant hit. E-values indicate the strength of a match between query sequence and target sequence, with a lower E-vale indicating a more significant match. For this study, an E-value of e^{-20} was chosen as a cut-off for significance. Values higher than this (i.e., approaching zero) were considered as less significant.

IV. Results

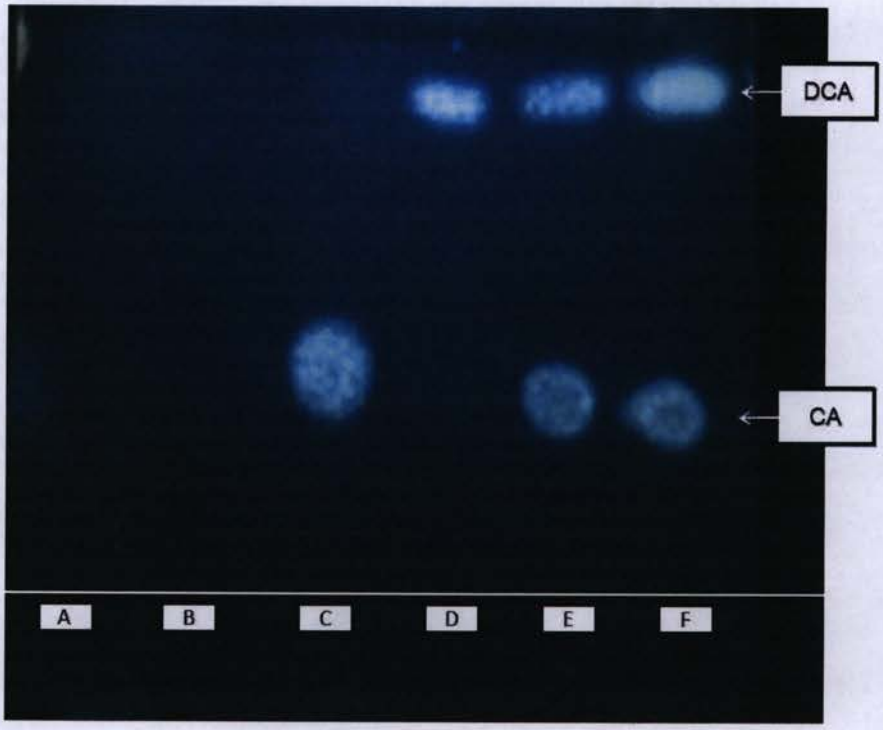
TLC analyses of Bile Acid Standards in Methanol

Previous experiments that showed the 7α-dehydroxylation activity of *C. scindens* were carried out in very rich nutritive medium like BHI [18, 32, and 73], so growth experiments followed by thin-layer chromatography (TLC) analyses to determine whether 7α-dehydroxylation of primary bile acids can also occur under defined conditions were carried out in this study to assess whether growth conditions of restrictive nutritional supplies will affect the bile acid metabolism of *C. scindens*.

Before performing experiments with *C. scindens*, the TLC procedure was standardized. CA and DCA standards of various concentrations ranging from 0.1 mM to 1 mM and from 0.01 mM to 0.1 mM were prepared and spotted on TLC plates and ran in a solvent system consisting of ethylacetate:cyclohexane: glacial acetic acid – 23: 7: 3 ml. The first TLC standardization consisted of 4 sample spots and a blank spot. The 4 samples were 1 mM CA standards, 1 mM DCA standards, 1 mM CA/DCA standards. The 1 mM CA/DCA sample was spotted twice for duplication.

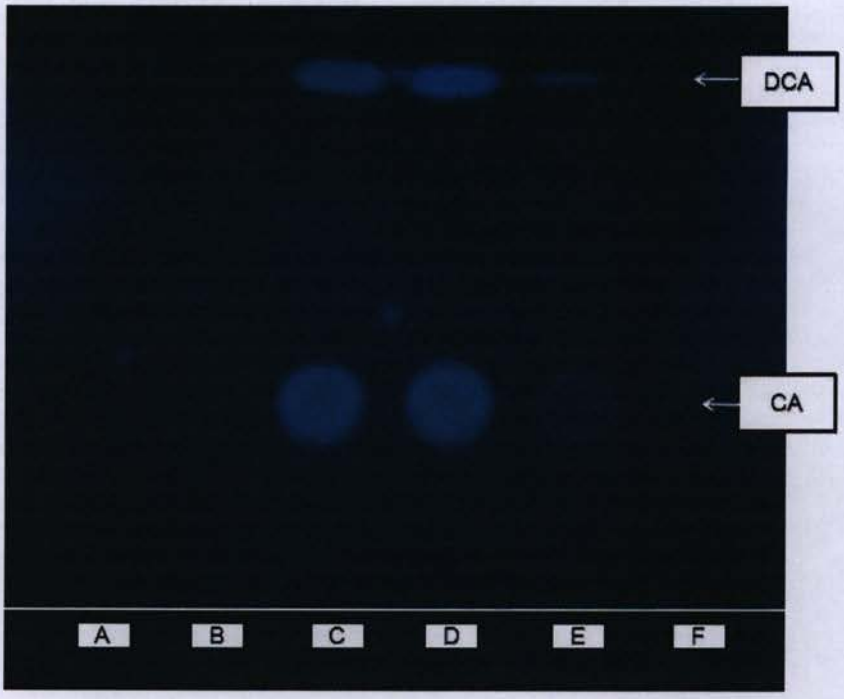
Results indicated that separation of the standards were of good quality under these conditions (Figure 8). Following this experiment, CA and DCA standards of various concentrations were run using the same technique to determine if it was possible to deduce the concentration of DCA being converted through 7α-dehydroxylation of primary bile acids based on differences in spot intensities. TLC with 2 serial 10-fold dilutions of CA/DCA starting at 1 mM concentration was run using the same standard procedure. The spot for 0.1 mM CA/DCA was faint whereas the spot for 0.01 mM CA/DCA standards was almost invisible (Figure 9). Hence to find out at what concentration the spots for CA/DCA standards became invisible (undetectable), different samples with different concentrations for CA/DCA were made.

To further refine the level of detection under these testing conditions, CA/DCA standards ranging from 0.1 mM to 1 mM and from 0.01 mM to 0.1 mM were prepared in methanol. Results showed that as the concentration decreased from 1 mM to 0.1 mM, the intensity of the spots also decreased. Thus the spot for 1 mM CA/DCA standard was the brightest and for the 0.1 mM CA/DCA standard was the faintest (Fig. 10). At the same time, when the concentrations increased from 0.01 mM to 0.1mM, the intensity of the spot also increased. Thus when CA/DCA is present in 0.01 mM concentration or less it becomes difficult to detect its presence making. Consequently, 0.02 mM was determined to be the threshold concentration for detection. It is interesting to notice that in both the cases (0.1-1 mM and 0.01- 0.1 mM), the medium concentration 0.6 mM and 0.06 mM is the brightest (Fig. 11).



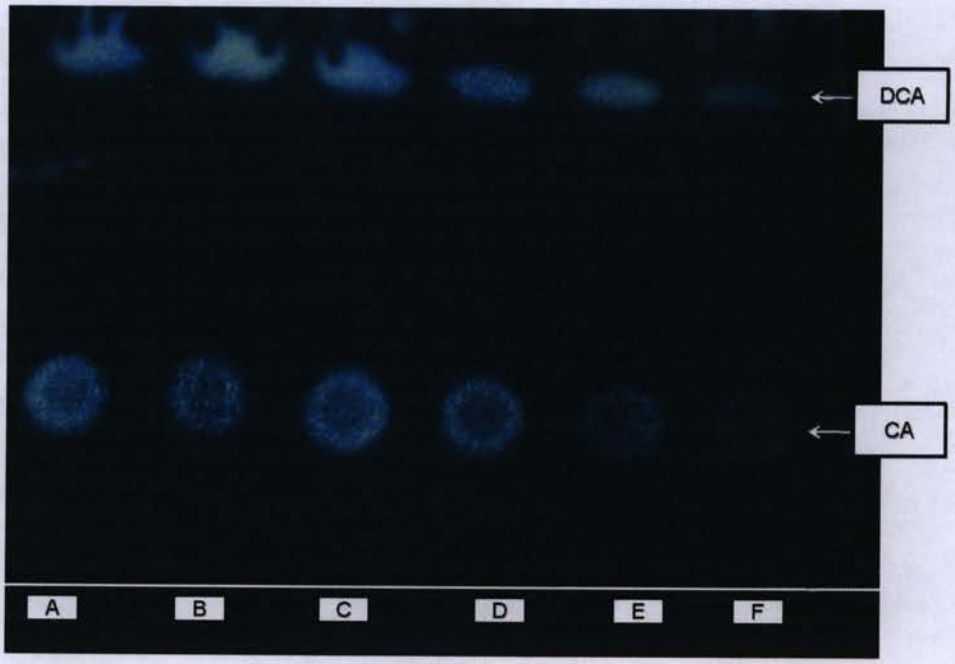
A: Blank Spot
B: MeOH
C:1 mM CA standards in MeOH
D: 1 mM DCA standards in MeOH
E: 1 mM CA/DCA standards in MeOH
F: 1 mM CA/DCA standards in MeOH

Fig. 8. TLC analysis of 1 mM bile acid standards prepared in methanol. The solvent system used: Ethyl acetate:cyclohexane:glacial acetic acid (23:7:3). Abbreviations: MeOH, methanol; CA, cholate; and DCA, deoxycholate.



A: Blank Spot
B: MeOH
C:1 mM CA/DCA standards in MeOH
D: 1 mM CA/DCA standards in MeOH
E: 0.1 mM CA/DCA standards in MeOH
F: 0.01 mM CA/DCA standards in MeOH

Fig. 9. TLC analysis of bile acid standards (1 mM, 0.1 mM and 0.01 mM) prepared in methanol. 50 µl of sample was spotted on a 20 x 20 non UV aluminum backed TLC plate. The modified solvent system used: Ethylacetate: cyclohexane: glacial acetic acid: chloroform – (18: 7: 3: 5) ml. The plate was charred using a charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: MeOH, methanol; CA, cholate; and DCA, deoxycholate.



A: 1 mM CA/DCA standards in MeOH
B: 0.8 mM CA/DCA standards in MeOH
C: 0.6 mM CA/DCA standards in MeOH
D: 0.4 mM CA/DCA standards in MeOH
E: 0.2 mM CA/DCA standards in MeOH
F: 0.1 mM CA/DCA standards in MeOH

Fig. 10. TLC analysis of 1 to 0.1 mM bile acid standards prepared in methanol. 50 µl of sample was spotted on a 20 x 20 non UV aluminum backed TLC plate. The solvent system used: Ethylacetate: cyclohexane: glacial acetic acid – (23: 7: 3) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: MeOH, methanol; CA, cholate; and DCA, deoxycholate.

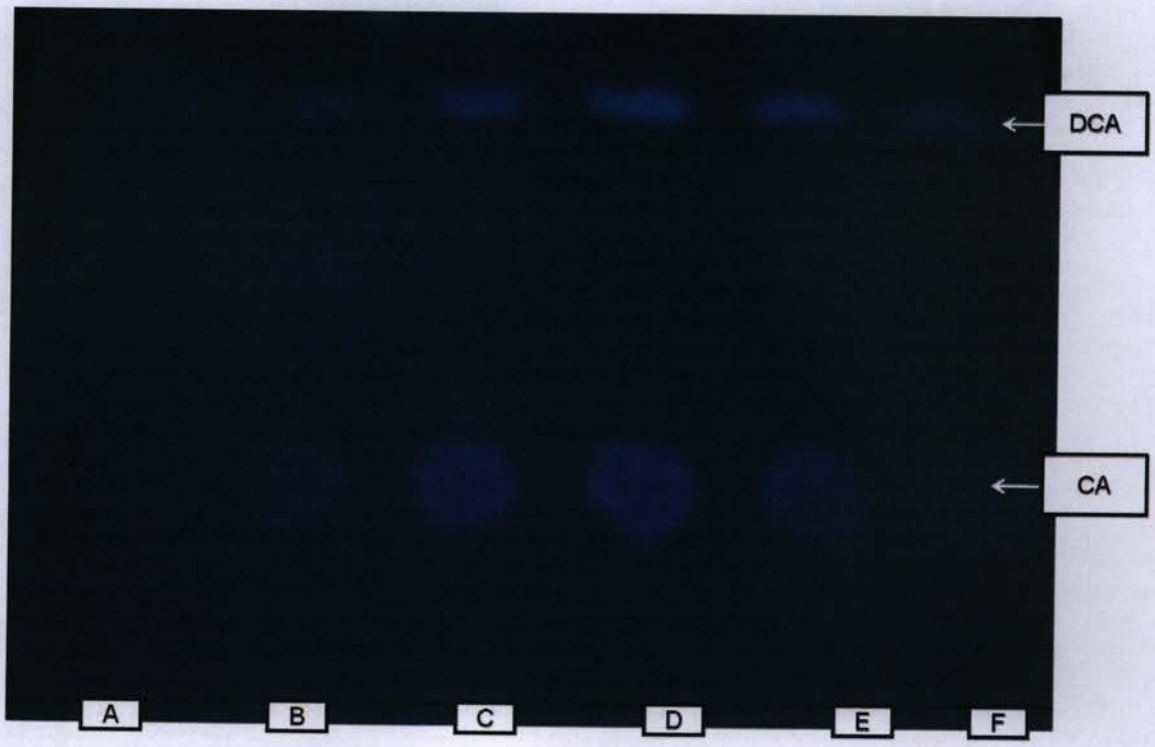
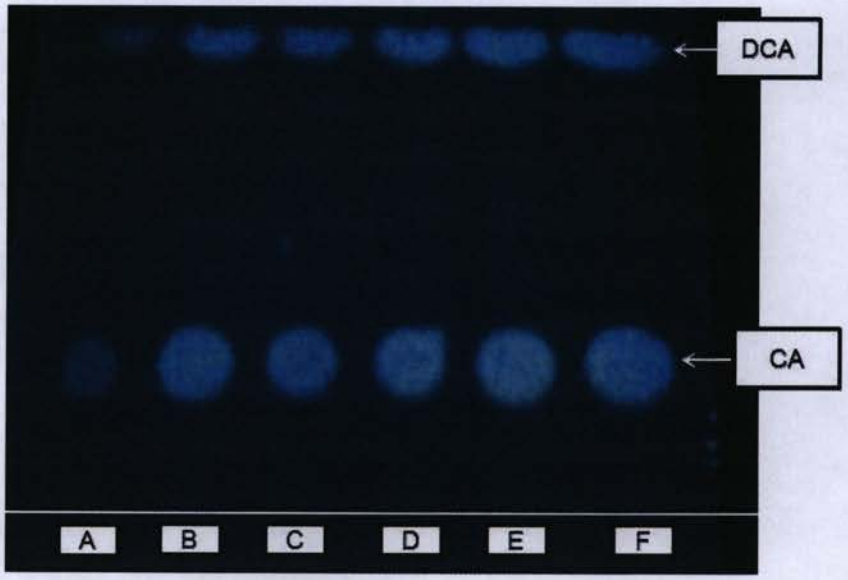


Fig. 11. TLC analysis of 0.01 to 0.1 mM bile acid standards prepared in methanol. 50 µl of sample was spotted on a 20 x 20 non UV aluminum backed TLC plate. The solvent system used: Ethylacetate: cyclohexane: glacial acetic acid – (23: 7: 3) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: MeOH, methanol; CA, cholate; and DCA, deoxycholate.

TLC Analyses of Bile Acid Standards Extracted from Culture Media

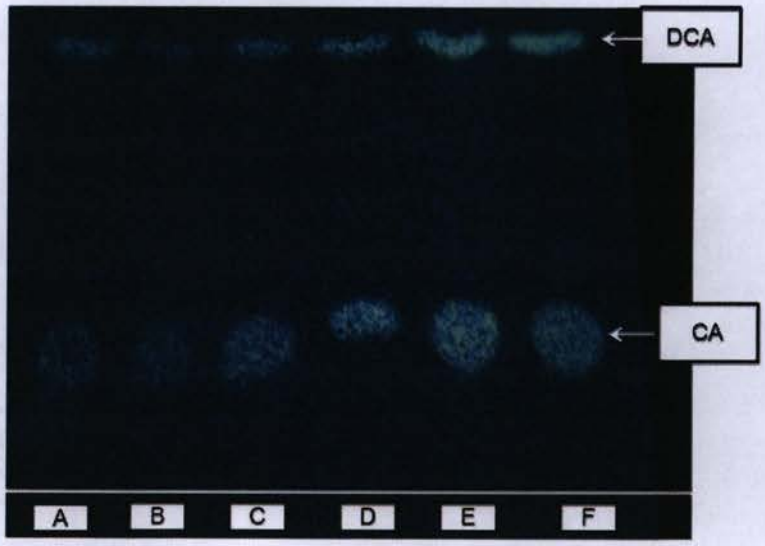
Once the procedure was standardized using methanol standards and also the threshold concentration for detection was identified, the next step was to run the CA/DCA standards extracted from the defined medium to check if there was any "impurity" which could potentially affect the separation pattern present in the medium or not. Testing standards of CA/DCA were prepared ranging from 0.1 to 1 mM as well as from 0.01 to 0.1 mM and then added to defined medium. For the CA/DCA standards ranging from 0.1 to 1 mM in concentration extracted from DM (Fig. 12), only the intensity for the 0.1 mM spot is the faintest whereas the intensity for all the remaining spots is more or less the same, unlike the methanol standards where a gradual increase in intensity with concentration was observed. For the concentrations 0.01 to 0.1 mM, the same gradual increase in intensity for the spots with increasing concentration was noticed as for standards prepared in methanol (Fig. 13). Even though there is a gradual increase in intensity of spots, the spots for concentrations 0.08 and 0.1 m appear to have same intensity. This suggested that in defined medium when CA/DCA are present in concentrations ranging between 0.1 mM to 1 mM or between 0.08 to 0.1 mM, the CA/DCA concentrations may be difficult to approximate due to a lack of assay sensitivity.



A: 0.1 mM CA/DCA standards in DM
B: 0.2 mM CA/DCA standards in DM
C: 0.4 mM CA/DCA standards in DM
D: 0.6 mM CA/DCA standards in DM
E: 0.8 mM CA/DCA standards in DM
F: 1.0 mM CA/DCA standards in DM

Note: DM = DM + CVS + CAAS

Fig. 12. TLC analysis of 0.1 to 1 mM bile acid standards prepared in defined medium (DM). 50 µl of sample was spotted on a 20 x 20 non UV aluminium backed TLC plate. The solvent system used: Ethylacetate: cyclohexane: glacial acetic acid – (23: 7: 3) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: DM, defined medium; CVS, complete vitamin solution; CAAS, complete amino acid solution; CA, cholate; and DCA, deoxycholate.



A: 0.01 mM CA/DCA standards in DM
B: 0.02 mM CA/DCA standards in DM
C: 0.04 mM CA/DCA standards in DM
D: 0.06 mM CA/DCA standards in DM
E: 0.08 mM CA/DCA standards in DM
F: 0.1 mM CA/DCA standards in DM

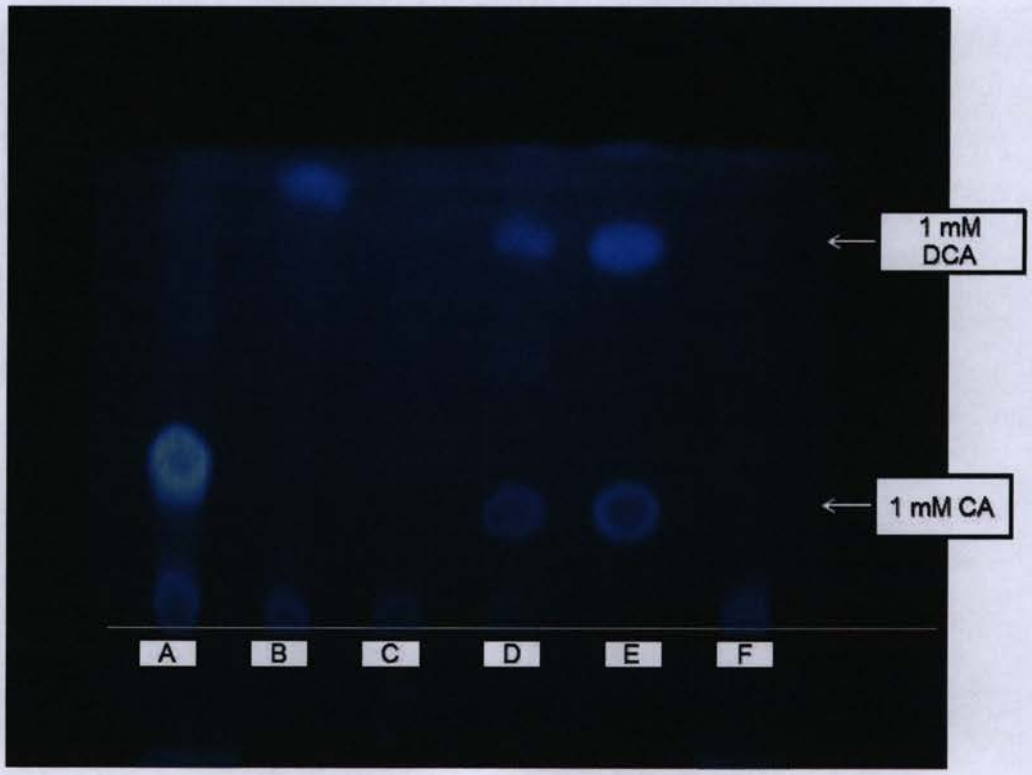
Note: DM = DM + CVS + CAAS

Fig. 13. TLC analysis of 0.01 to 0.1 mM bile acid standards prepared in defined medium (DM). 50 µl of sample was spotted on a 20 x 20 non UV aluminium backed TLC plate. The solvent system used: Ethylacetate: cyclohexane: glacial acetic acid – (23: 7: 3) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: DM, defined medium; CVS, complete vitamin solution; CAAS, complete amino acid solution; CA, cholate; and DCA, deoxycholate.

TLC- Based Detection of Bile Acid Dehydroxylation by *C. scindens* VPI 12708 Grown in BHI and DM with 1 mM CA

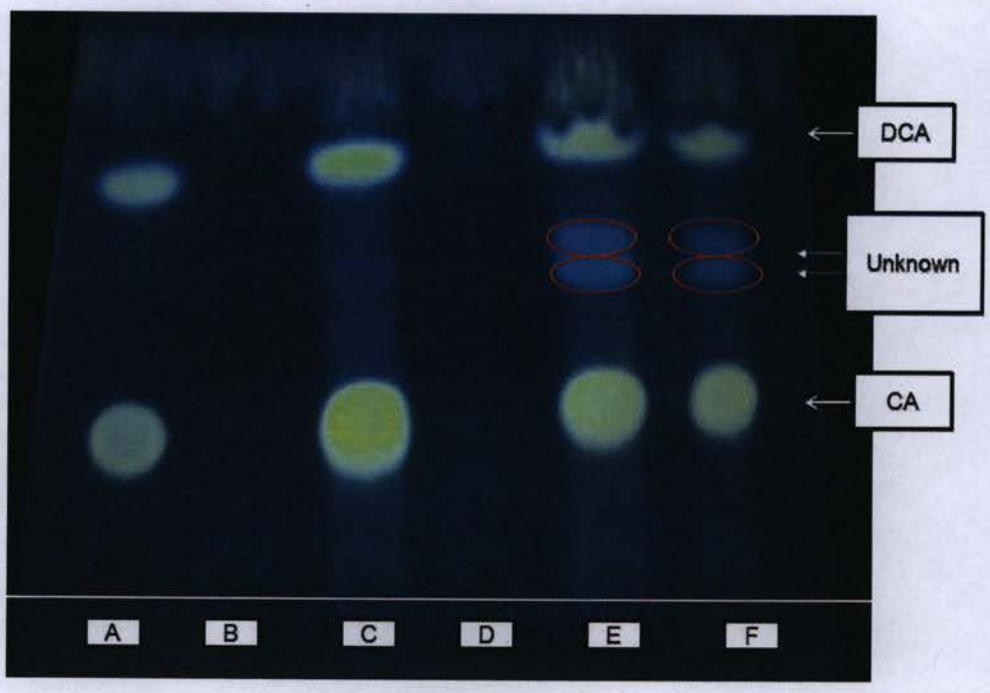
As the main goal of the first objective was to see if *C. scindens* VPI 12708 dehydroxylated primary bile acids to secondary bile acids under defined culture conditions or not, initial experiments were done to first verify that *C. scindens* VPI 12708 was competent in bile acid dehydroxylation. Since past studies have exclusively used rich nutritive culture conditions to demonstrate bacterial dehydroxylation of bile acids, *C. scindens* was grown in BHI with 1 mM cholate. Measurements using OD (600nm) showed that *C. scindens* grew well with 1 mM CA in BHI. Once the growth of *C. scindens* was observed in BHI with 1 mM CA, *C. scindens* was then cultivated in DM with 1 mM CA in the same way. After three subsequent passages, growth was comparatively higher in BHI supplemented with 1 mM CA. In DM with 1 mM CA, growth was also well supported (see growth data for this experiment in Fig. 17).

After growing *C. scindens* VPI 12708 in BHI and DM supplemented with 1 mM CA, the media was extracted for TLC by the extraction technique described in the Materials and Methods section. *C. scindens* dehydroxylated CA and converted it to the secondary bile acid DCA under both nutritive rich culture conditions (BHI, Fig. 14) and under defined culture conditions with or without vitamin and amino acid supplementation (Figures 15 and 16, respectively). In DM supplemented with vitamin and amino acids, two additional spots were detected, suggesting additional products were formed during the dehydroxylation process. In contrast, when *C. scindens* dehydroxylated CA and converted it to DCA in BHI (Fig. 14) and in DM without supplementation, only a single, faint spot was observed. However, it is impossible to determine based on TLC results whether the two spots from BHI and DM without supplementation were representing the same compound(s). These unknown compounds may be by-products of dehydroxylation or impurities that co-precipitated from the growth media during sample preparation.



A: 1 mM CA in Sterile BHI
B: 1 mM DCA in Sterile BHI
C: BHI + *C. scindens*
D: BHI + 1 mM CA + *C. scindens*
E: 1 mM CA/DCA standards in MeOH
F: Sterile BHI

Fig. 14. TLC analysis of bile acid dehydroxylation by *C. scindens* VPI 1208 in brain heart infusion medium (BHI). 50 µl of sample was spotted on a 20 x 20 non UV aluminum backed TLC plate. The solvent system used: Ethylacetate: cyclohexane: glacial acetic acid – (23: 7: 3) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: BHI, brain heart infusion broth; MeOH, methanol; *C. scindens*, *Clostridium scindens*; CA, cholate; and DCA, deoxycholate.



A: 1 mM CA/DCA standards in MeOH
B: Sterile DM+CVS+CAAS
C: 1 mM CA/DCA standards in DM+CVS+CAAS
D: DM+CVS+CAAS + *C. scindens*
E: DM+CVS+CAAS + 1 mM CA + *C. scindens*
F: DM+CVS+CAAS + 1 mM CA + *C. scindens*

Fig. 15. TLC analysis of bile acid dehydroxylation by *C. scindens* VPI 12708 in defined medium (DM). 50 µl of sample was spotted on a 20 x 20 non UV aluminum backed TLC plate. The solvent system used: Ethylacetate: cyclohexane: glacial acetic acid – (23: 7: 3) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: MeOH, methanol; DM, defined medium; CVS, complete vitamin solution; CAAS, complete amino acid solution; *C. scindens*, *Clostridium scindens*; CA, cholate; and DCA, deoxycholate.

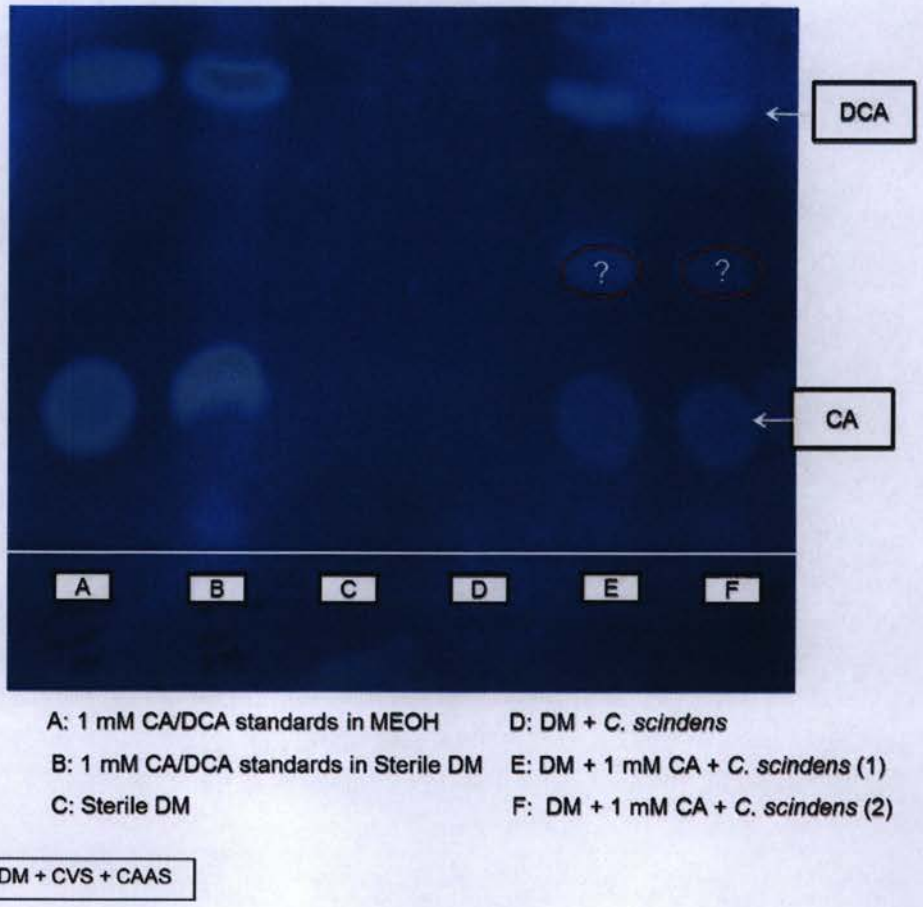


Fig. 16. TLC analysis of bile acid dehydroxylation by *C. scindens* VPI 12708 in defined medium (DM) supplemented with 1 mM CA. 50 µl of sample was spotted on a 20 x 20 non UV aluminum backed TLC plate. The modified solvent system used: Ethylacetate: cyclohexane: glacial acetic acid: chloroform – (18: 7: 3: 5) ml. The plate was charred using charring agent consisting of: Methanol: 150 ml; Water: 150 ml; Conc. H_2SO_4: 10 ml; $MnCl_2.4H_2O$: 1 gm. The plate was baked in an oven at 150°F for 15 minutes. After the plates cooled, they were observed under long range UV light. Abbreviations: MeOH, methanol; DM, defined medium; CVS, complete vitamin solution; CAAS, complete amino acid solution; *C. scindens*, *Clostridium scindens*; CA, cholate; and DCA, deoxycholate.

Resolving the Vitamin and Amino Acid Requirements of *C. scindens* VPI 12708

The next goal of the project was to resolve the nutritional requirements of *C. scindens*. To complete this objective, *C. scindens* was first grown in different nutritional media to see which serves as the best for the growth of *C. scindens*. When grown in different growth culture conditions, as the medium changed from being a very rich nutritive medium (BHI) to an undefined medium to a defined medium, the organism's optimal growth decreased (Fig. 17 and 18). *C. scindens* VPI 12708 did the best in BHI and the worst in UM. Usually UM on its own is known to be nutritionally sufficient for the growth of most anaerobic bacteria; however, *C. scindens* interestingly did poorly on UM alone. Thus, *C. scindens* was grown in UM alone and also in UM supplemented with CVS and CAAS, which, after 3 consecutive passages, supported growth better than UM alone (Fig. 18). Once the need to supplement UM with CVS and CAAS was figured out, the next task was to determine if using filter sterilized (F.S.) CVS and CAAS versus autoclaved (AV) CVS and CAAS made any difference to the growth of *C. scindens* in UM since vitamins and amino acids are usually heat labile. Relative to growth, *C. scindens* grew better in UM when autoclaved CVS and autoclaved CAAS were added (Fig. 18). The reason could be that the heat (autoclaving) helped to solubilize the vitamins and amino acids or generated breakdown products that favored better growth of *C. scindens* VPI 12708.

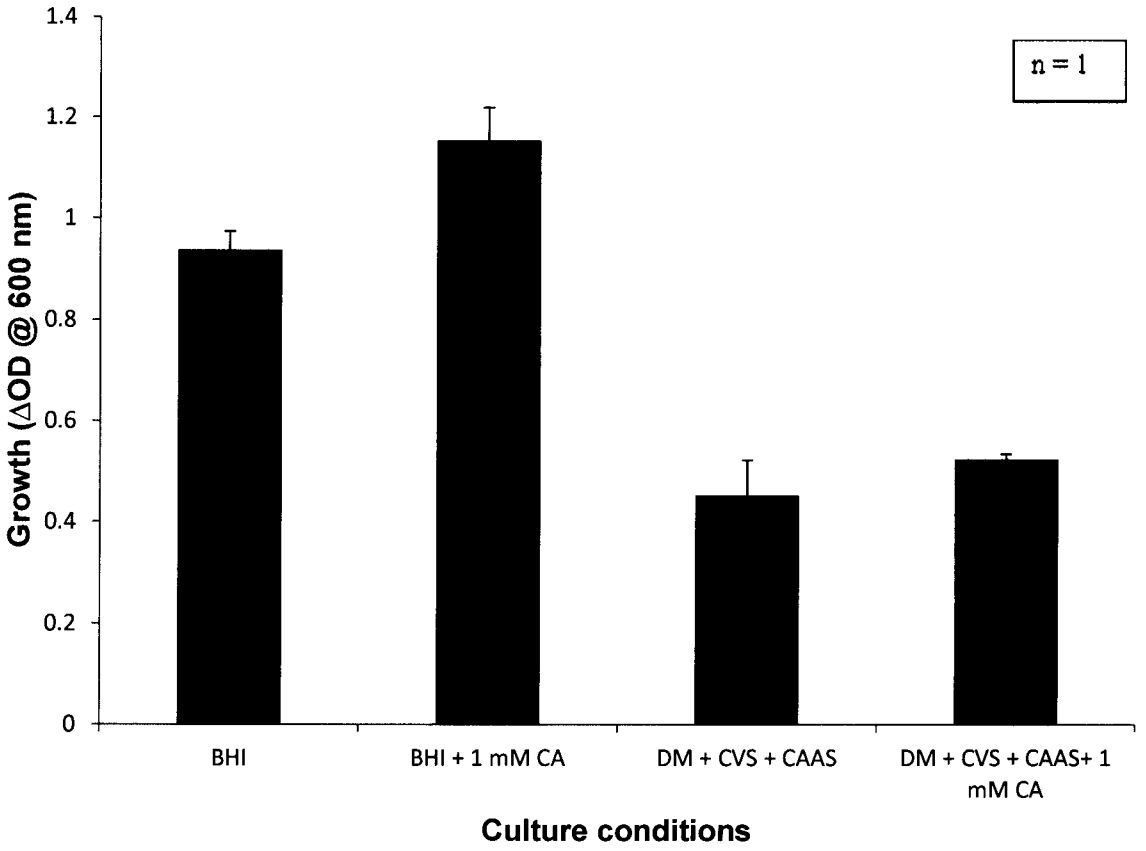


Fig. 17. Growth of *C. scindens* VPI 12708 in BHI and DM in the presence and absence of 1 mM Cholate. Data represents 3 replicates from a single trial and the vertical bars depict standard deviation.

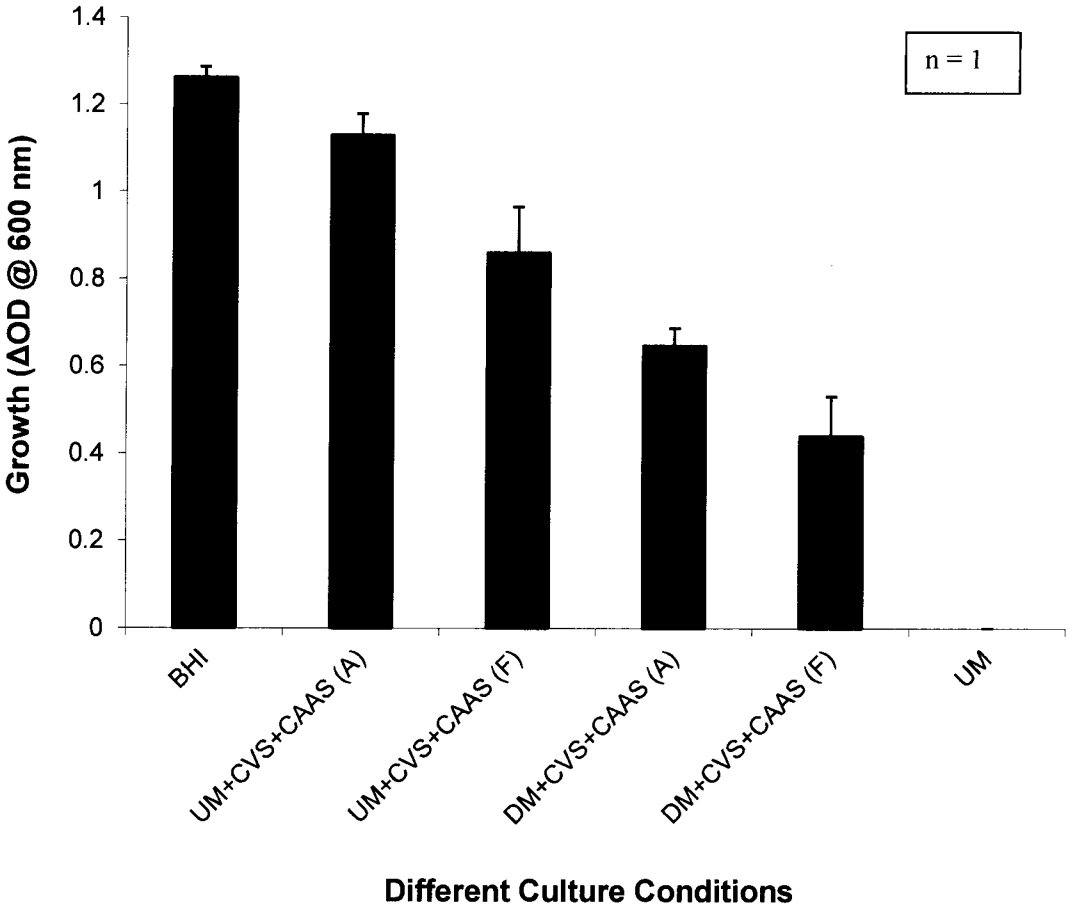


Fig. 18. Growth of *C. scindens* VPI 12708 under different culture conditions. Values represent the means of triplicate tubes after third passage. Data represents 3 replicates from a single trial and the vertical bars depict standard deviation.

Initially, to determine if *C. scindens* VPI 12708 possessed a growth-factor requirement for vitamins and amino acids, growth of the organism was evaluated under the following culture conditions: DM + CVS + CAAS; DM + CVS (no CAAS); DM + CAAS (no CVS); DM (no CVS or CAAS). Growth was measured over time until the maximum OD was reached, and cultures were maintained by sequentially transferring them to their respective media. After 3 sequential passages in a given supplement-deficient medium (DM + CVS; DM + CAAS; and DM), if culture OD (600 nm) was ≤ 0.05, the medium was considered non-growth supportive and hence indicated that a specific growth factor (vitamin or amino acid or both) was required by *C. scindens* VPI 12708 for growth.

Based on the above experimental conditions, *C. scindens* VPI 12708 required both vitamins and amino acids for its growth in DM because when either or both of these were omitted from the DM, *C. scindens* VPI 12708 grew poorly and essentially stopped growing after the first passage (Fig. 19).

Once the need for vitamins and amino acids for growth of *C. scindens* was observed, the nature of the specific vitamin(s) requirement of *C. scindens* VPI 12708 was determined by using the leave-one-out technique (see Materials and Methods). After 3 sequential passages in a given vitamin-deficient medium (DM + CAAS + CVS-1), if culture OD (600 nm) was ≤ 0.05, the medium was considered non-growth supportive and hence indicated the specific vitamin (the one missing in the CVS-1 solution) that was required by *C. scindens* VPI 12708 for growth. Based on this experiment, *C. scindens* VPI 12708 required biotin, pyridoxal, pantothenate and riboflavin for its growth because when these four vitamins were absent from the growth medium, there was little to no growth observed (Fig. 20).

Once the types of vitamins required for the growth of *C. scindens* VPI 12708 were resolved, several modified vitamin mixes (consisting of different combination of biotin, pyridoxal,

pantothenate and riboflavin) were prepared for further validation. Five different sterile modified vitamin solutions were prepared as described in the Materials and Methods section for CVS, except one of the mixes had the four essential vitamins and the remaining four modified vitamin solutions were prepared by leaving one of the four essential vitamins out. Unexpectedly, the results from this validation experiment did not agree with results from previous experiments, meaning the vitamins initially found to be required for growth were now different. Instead of biotin, pyridoxal, pantothenate and riboflavin, only pantothenate and riboflavin were now required for growth by *C scindens* VPI 12708. Initially when grown in defined medium supplemented with CVS-biotin, after the second passage growth of *C. scindens* started to decline and in the third passage there was no growth of *C. scindens* in defined medium with CVS-biotin. But when using the defined medium supplemented with modified vitamin solution minus biotin, *C. scindens* kept growing until the third passage with no fall out. As biotin was deemed as one of the important vitamins required for growth, its absence from the medium should not support growth of *C. scindens*. Instead *C. scindens* kept growing in the defined medium supplemented with modified vitamin solution minus biotin. Similarly when grown in defined medium supplemented with CVS-pyridoxal earlier, *C. scindens* barely grew until the second passage and when transferred to the third passage, there was no growth of *C. scindens* in defined medium with CVS-pyridoxal. Again when using defined medium supplemented with modified vitamin solution minus pyridoxal, *C. scindens* should not be growing in the medium lacking pyridoxal but the growth data showed results that were opposite to the expected outcome.

To address these conflicting results, a series of experiments were done. First, the original culture of *C. scindens* and a new culture of *C. scindens* (both cultures were provided by Dr. Doerner at Western Kentucky University) were grown in DM supplemented with modified vitamin solution to see if the conflict was due to original culture of *C. scindens* VPI 12708. I stopped using the original culture provided by Dr. Doerner and switched to a new culture as it

was difficult getting reproducible results using the "old culture". I could get reproducible results using the other culture and hence continued with experiments using the second culture. The second culture was called the "new culture." *C. scindens* from the old culture did better compare to the new one (Fig. 21). One of the reasons could be that the old culture was a more "stable" culture whereas the new culture might have undergone some kind of mutation. *C. scindens* was also grown in DM supplemented with an old batch of (F.S.) CVS and also in DM supplemented with a new batch of (F.S.) CVS to check if the CVS had a role to play in giving conflicting results. The new batch of CVS was prepared by making the solution as described in materials and methods but after the solution was prepared, it was placed in hot water bath at 60°C for 15 minutes so that the vitamins will get more solubilized. *C. scindens* grew better in the DM supplemented with a new batch of CVS (Fig. 22).

The confirmation experiment for resolving vitamin requirements for *C. scindens* VPI 12708 was done again using all freshly prepared modified solutions. After three transfers, *C. scindens* VPI 12708 grew very well in all the different culture conditions (Fig. 23). As no decline in growth of *C. scindens* VPI 12708 was observed in either of the growth conditions after third passage as it should have been as each one of those MVS were missing on an essential vitamin, *C. scindens* VPI 12708 was continued for another passage to see if there was a decline in growth. After the fourth passage, there was less to no growth seen in riboflavin and pantothenate (Fig. 23) but *C. scindens* continued to grow in biotin and pyridoxal. Hence the two main vitamins required by *C. scindens* for growth are pantothenate and riboflavin.

Resolving amino acid requirements was equally important as resolving vitamin requirements of *C. scindens*. The amino acids were divided into six different groups since amino acids within a specific group may be interconverted by bacteria. See Materials and Methods for the different groups. Six different amino solutions (100 ml each) were prepared as described in Materials and Methods for the CAAS, except that each solution was made deficient in a particular amino acid

group by omitting the addition of individual solutions containing the amino acids that were specific to that group. After 3 sequential passages in a given amino acid group-deficient medium (DM + CVS + CAAS-1), results showed that the amino acids from the aromatic group were needed for the growth of *C. scindens* (Fig. 24).

Once the amino acids group required for growth by *C. scindens* VPI 12708 was identified, the specific amino acid(s) in that group needed for growth was determined by the "leave-one-out technique". The required amino acid group solution was prepared by combining 5 ml of each of the individual amino solutions specific to that group and bringing the volume to 100 ml with RO water. The required group solution was also made deficient in one or more of the amino acids specific to that group by omitting the individual solutions for those amino acids. After 3 sequential passages in a given amino acid-deficient medium, results showed that tryptophan was the one which was required for the growth of *C. scindens* VPI 12708 (Fig. 25).

Once the vitamin and amino acid requirements were resolved, *C. scindens* was grown in DM supplemented with CVS + CAAS, MVS + CAAS, CVS + Tryptophan (Trp), MVS + Trp and, water to assess growth conditions for *C. scindens* VPI 12708. *C. scindens* grew as well in DM + CVS + Trp as it did in DM + CVS + CAAS (Fig. 26). Also, when compared to the growth of *C. scindens* in DM + MVS + CAAS, the growth of *C. scindens* in DM + CVS + Trp was better. This suggested that even though the DM was supplemented with CAAS and not CVS, CVS appeared to be more important for the growth of *C. scindens* than CAAS. This suggested that *C. scindens* required CVS for better growth under defined culture conditions whereas tryptophan alone, not CAAS, is sufficient for the growth of *C. scindens*.

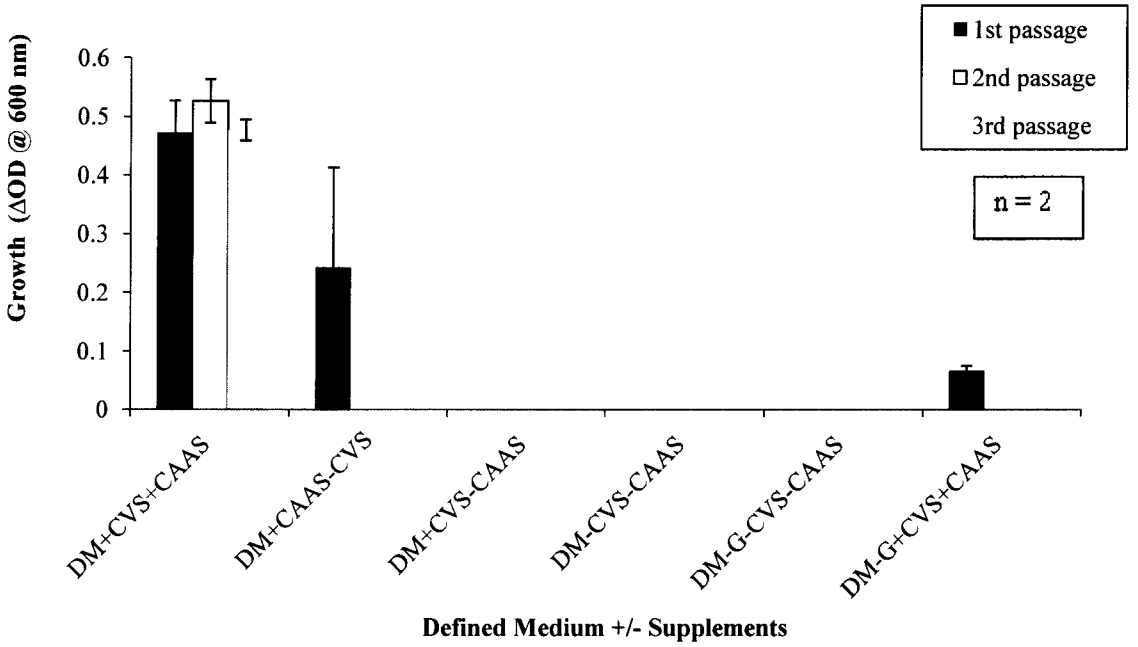


Fig. 19. Determination of the vitamins and amino acid requirements of *C. scindens* VPI 12708. Organism was grown in DM +/- supplements. n = 2 where n is the number of trials. In each trial duplicate tubes were used hence mean is of 4 tubes from 2 trials. The error bars represent standard deviation.

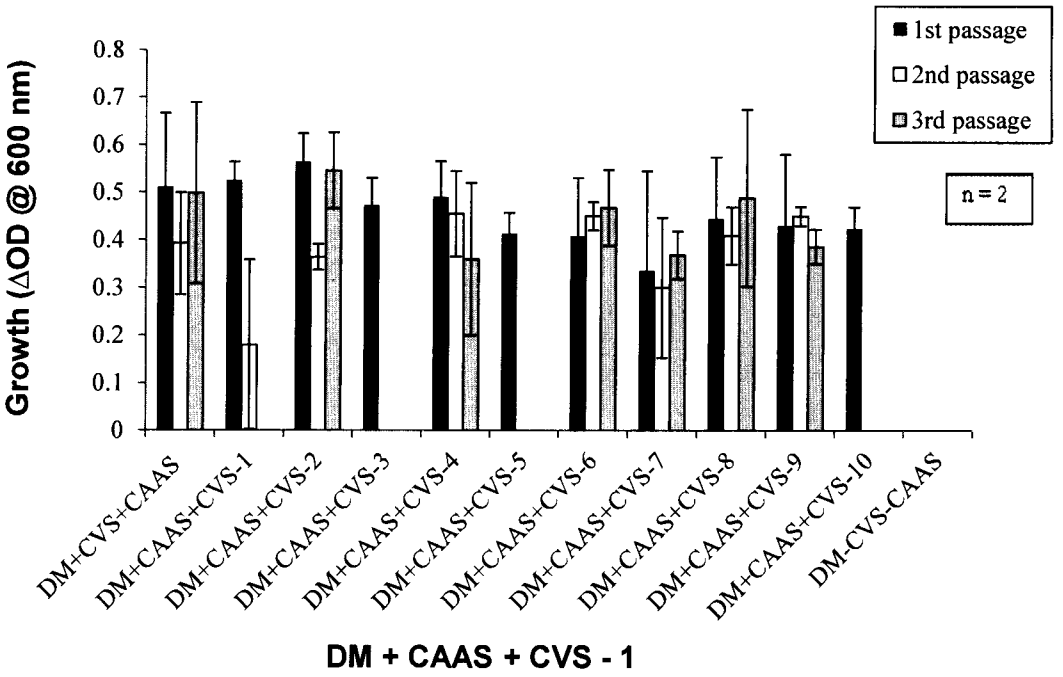


Fig. 20. The leave-one-out technique to determine the vitamin requirements of *C. scindens* VPI 12708. Organism was grown in DM + CAAS + CVS-1 vitamin. n = 2 where n is number of trials. In 1st trial duplicate tubes were used and in the second trial 1 tube was used hence mean is of 3 tubes. The error bars represent standard deviation.

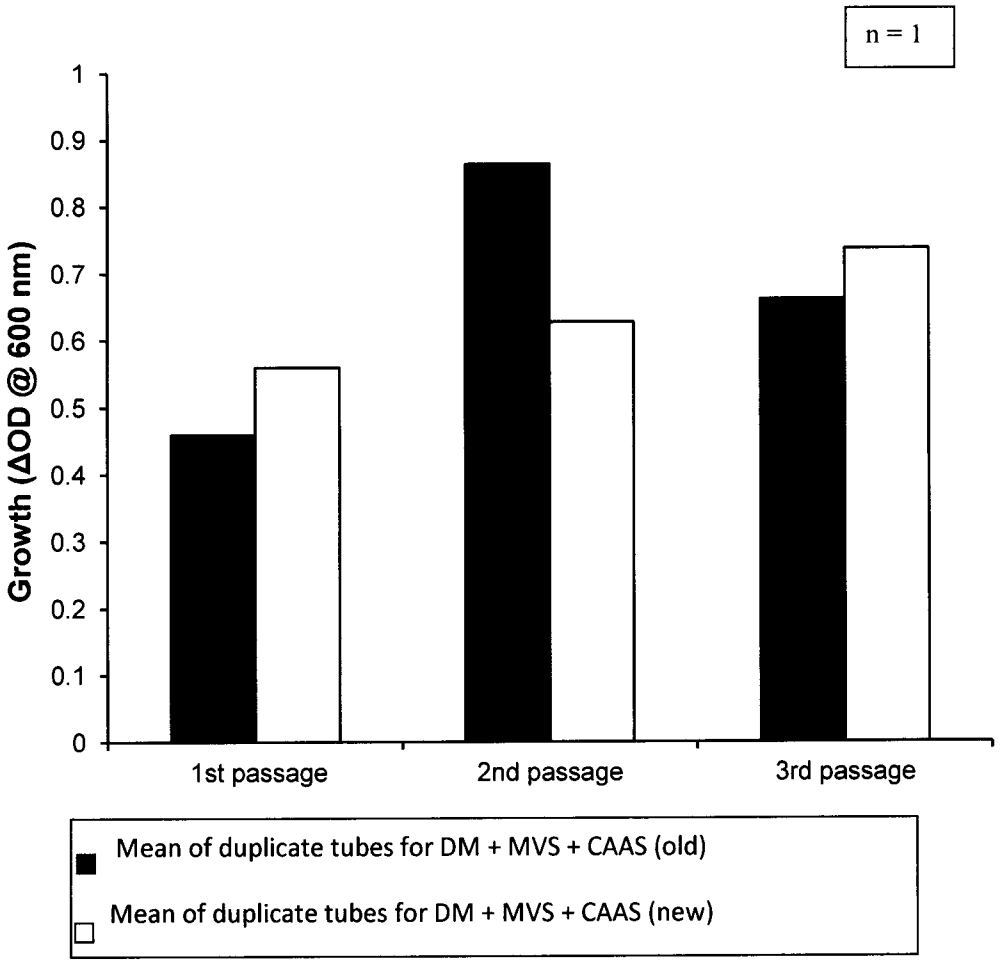


Fig. 21. Growth of *C. scindens* VPI 12708 in DM + CAAS + MVS inoculated with old and new cultures of *C. scindens* VPI 12708. n = 1 where n is the number of trials.

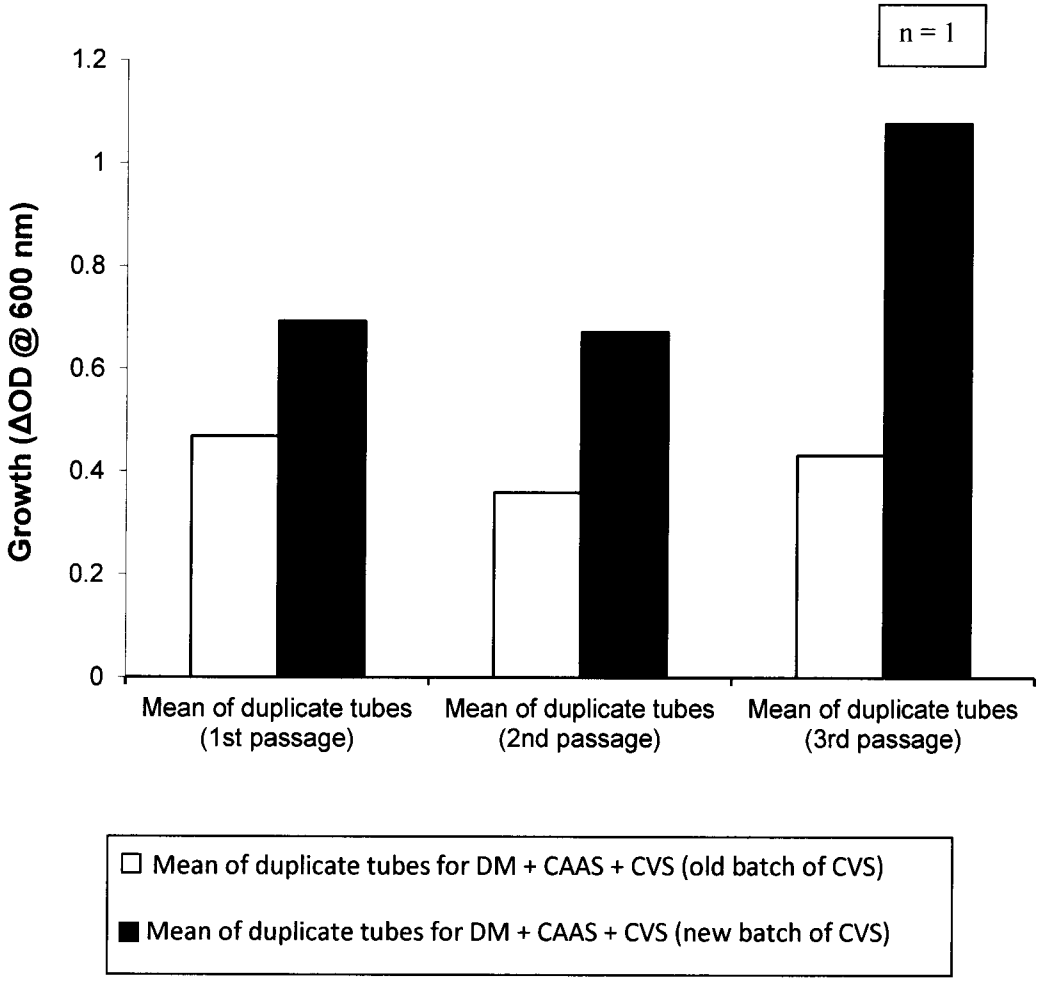


Fig. 22. Growth of *C. scindens* VPI 12708 in DM + CAAS supplemented with old or new batches of CVS. n = 1 where n is the number of trials.

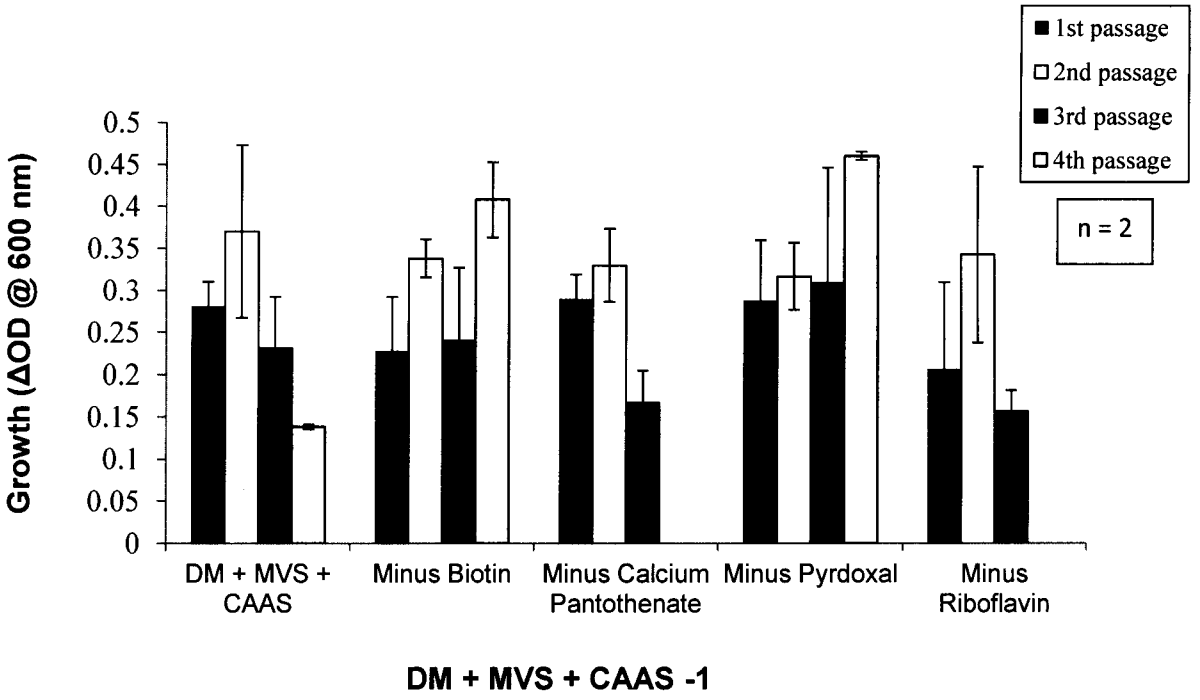


Fig. 23. Determination of the vitamin requirements of *C. scindens* VPI 12708 in DM supplemented with CAAS and MVS – 1 vitamin. n = 2 where n is number of trials and in each trial duplicate tubes were used hence mean is of 4 tubes in total from 2 trials. The error bars represent standard deviation.

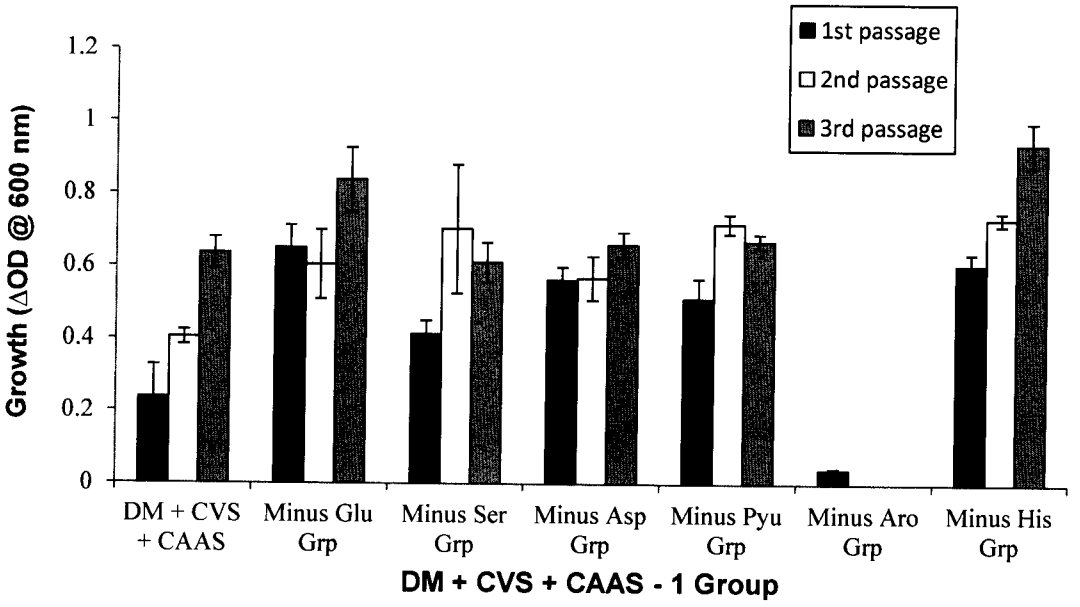


Fig. 24. Determination of the amino acid requirements for *C. scindens* VPI 12708. Organism was grown in DM supplemented CVS and CAAS – different groups of amino acids. n = 2 where n is the number of trials. In each trial duplicate tubes were used hence the mean is of 4 tubes from 2 trials. The error bars represent standard error. Note: For DM + CVS + CAAS n=1 hence duplicate tubes are only from 1 trial.

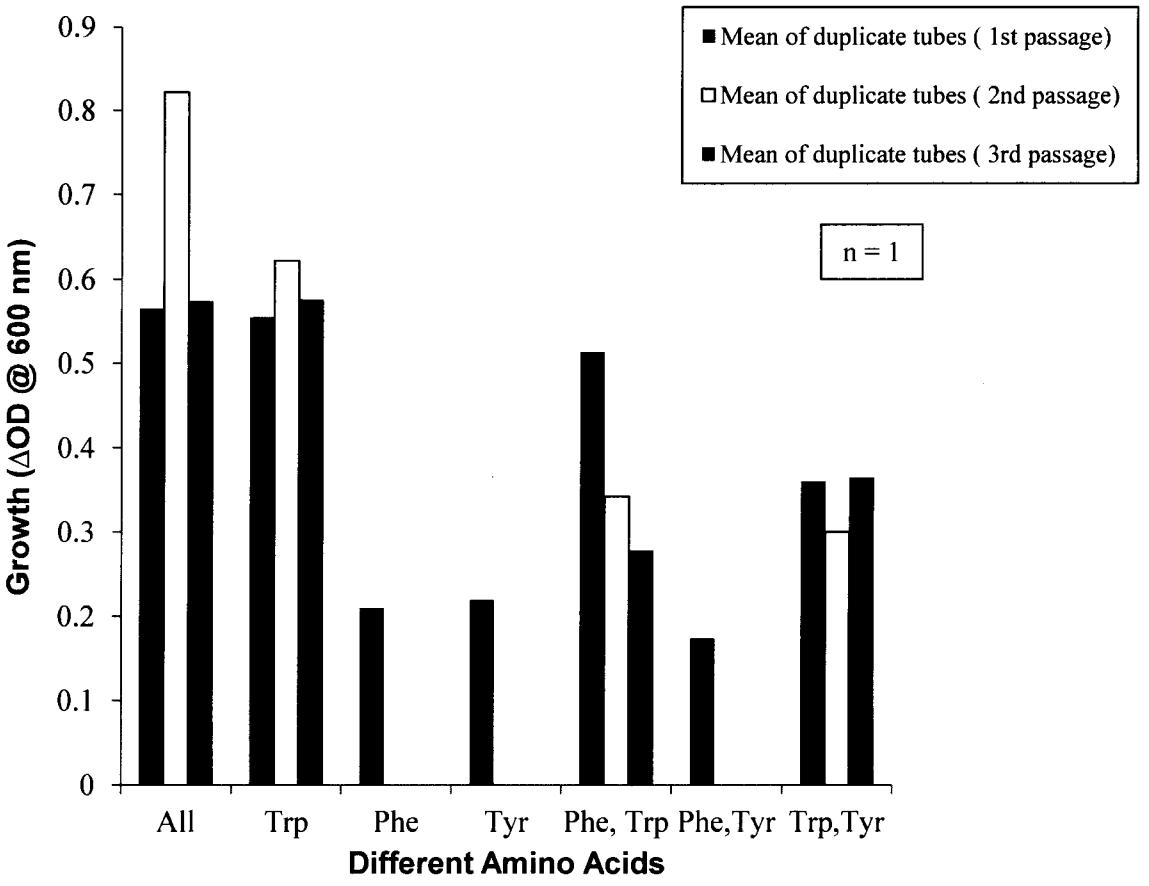


Fig. 25. Determination of the individual amino acids required for growth by *C. scindens* VPI 12708. Organism was grown in DM supplemented with CVS and with one or more the amino acids in the aromatic group. n = 1 where n is the number of trials. In each trial duplicate tubes were used hence the mean is of 2 tubes from 1 trial.

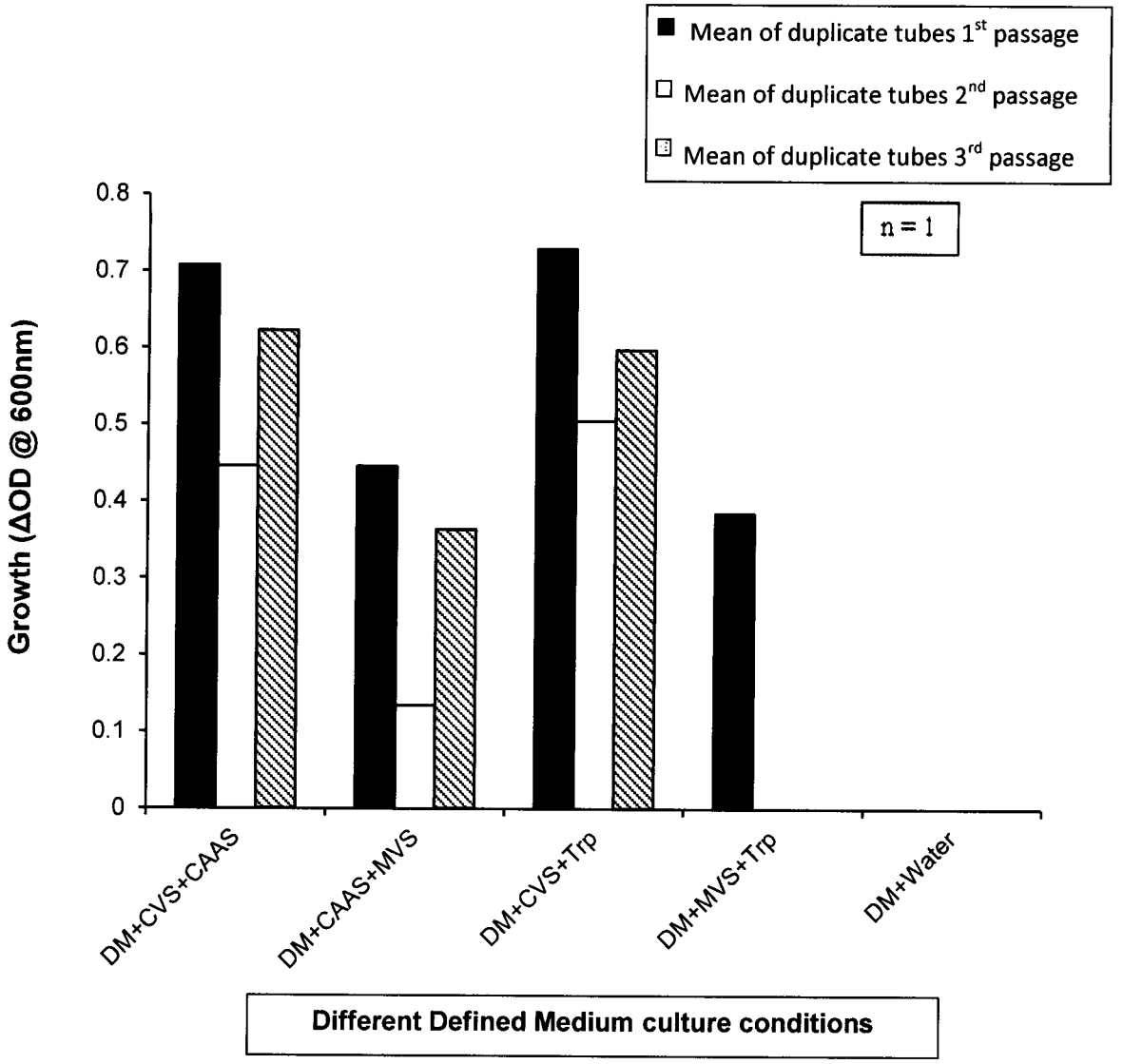


Fig. 26. Growth under different defined conditions for *C. scindens* VPI 12708. n = 1 where n is the number of trial. Each trial had duplicate tubes hence mean is of 2 tubes.

Results from BLAST search (Bioinformatics tools)

Once the vitamin and amino acid requirements were resolved, why those specific vitamins and amino acids were required and not synthesized by *C. scindens* itself was important to know. The reason could very probably be that *C. scindens* lacked the genes necessary for biosynthesis (anabolism) of these growth factors. To screen for the presence of the genes involved in the biosynthesis of the required vitamins and amino acids, BLAST analysis was performed as described in Materials and Methods using *E. coli* K-12 MG1655 as the reference organism for the complete pathways of different vitamins and amino acids elucidated so far.

After running the BLAST search and keeping the E-value - e^{-20} as the cut-off, the genes whose proteins showed similarity between *E. coli* K-12 substrain MG1655 and *C. scindens* are as follows:

Table 3. BLAST results with E-values for riboflavin (Rib), pyridoxal (Pdx), and biotin (Bio) proteins assumed to be present in *C. scindens* VPI 12708.

Protein name	Accession number in *E. coli*	Accession number in *C. scindens*	E - value
RibF	**NP_414566**	**i) CLOSCI_00410** **ii) CLOSCI_01811**	**$6e^{-55}$** **$4e^{-31}$**
SerC	**YP_025294**	**i) CLOSCI_00706**	**$3e^{-111}$**
EpD	**NP_418222**	**i) CLOSCI_01404** **ii) CLOSCI_01132**	**$8e^{-74}$** **$3e^{-71}$**
PdxA	**NP_414620**	**i) CLOSCI_01300**	**$9e^{-51}$**
PdxB	**NP_418127**	**i) CLOSCI_03743**	**$1e^{-23}$**
BioF	**YP_026248**	**i) CLOSCI_01570**	**$9e^{-52}$**
BioA	**YP_026247**	**i) CLOSCI_01767**	**$3e^{-48}$**
BioB	**NP_418096**	**i) CLOSCI_01529**	**$2e^{-52}$**
IlvC	**NP_418222.1**	**i) EDS08082.1**	**$1e^{-39}$**

Table 4. BLAST results with E-values for riboflavin (Rib), pantothenate (Pan) and tryptophan (Trp) proteins assumed to be absent in *C. scindens* VPI 12708.

Protein name	Accession number in *E. coli*	Accession number in *C. scindens*	E - value
RibA	**CAA42835**	**i) CLOSCI_01638**	**4.3**
RibB	**AAA69209**	**i) CLOSCI_03582**	**1.1**
RibD	**NP_414948**	**i) CLOSCI_00991** **ii) CLOSCI_02654**	**$3e^{-16}$** **$1e^{-06}$**
RibE	**NP_414949**	**i) CLOSCI_01009**	**4.5**
RibC	**NP_416179**	**i) CLOSCI_00966**	**0.43**
PanB	**NP_414676**	**i) CLOSCI_03163**	**0.82**
PanC	**AAC73244**	**i) CLOSCI_02235**	**0.060**
PanD	**NP_414673**	**i) CLOSCI_02857**	**0.88**
PanE	**NP_414959**	**i) CLOSCI_00705**	**1.0**
TrpD	**AAA57298**	**i) EDS05734.1** **ii) EDS06826.1**	**$7e^{-11}$** **$4e^{-07}$**
TrpE	**NP_415780**	**i) EDS08760.1**	**0.96**
TrpC	**NP_415778.2**	**i) EDS06272**	**0.87**
TrpA	**NP_4157761.1**	**i) EDS06591.1**	**0.28**
TrpB	**NP_415777.1**	**i) EDS07202.1**	**$4e^{-05}$**

From the BLAST results (see Table 4) it can be seen that *Clostridium scindens* VPI 12708 lacks the proteins whose genes code for the enzymes that are involved in the important steps in pathways for vitamin and amino acid biosynthesis. For example, in the pantothenate pathway, *pan*B encodes for the enzyme 3-methyl-2-oxobutanoate hydroxymethyltransferase which is required for the first committed step in the pathway. Hence, as *C. scindens* lacked the very first enzyme in this pathway, it would be essentially impossible for *C. scindens* to synthesize pantothenate on its own. Similarly, *C. scindens* appeared to lack most, if not all, of the other important genes from this pathway as well as genes necessary for riboflavin and tryptophan biosynthesis. For biotin and pyridoxal biosynthesis, all of the genes are present except for two important genes in each pathway. For biotin pathway, the two important genes missing from *C. scindens* were *bio*D and *bio*H (results not shown) whereas the other three genes *bio*F, *bio*A and *bio*B (see table 3) were present in *C. scindens*. These genes (*bio*F, *bio*A and *bio*B) are involved in the early steps of biotin biosynthesis whereas the other missing genes are involved in the latter stages. Similarly for pyridoxal, the genes *pdx*J and *pdx*H (results not shown) code for the enzymes involved in the last steps of pyridoxal biosynthesis which are important in the pathway and were missing from *C. scindens*, whereas *pdx*A and *pdx*B which were present are involved in the early steps of the pathway. Given the absence of genes essential for the synthesis of biotin and pyridoxal, it was surprising that these two vitamins were not required for growth. Some possibilities exist to account for these differences and will be discussed in the next section.

V. Discussion

The human body synthesizes bile acids from cholesterol in the liver. Bile acids produced by the liver are converted into secondary bile acids by the action of microorganisms in the intestine via the process of dehydroxylation. Many species from the *Clostridia* family are capable of dehydroxylating primary bile acids. One of the many microorganisms engaged in dehydroxylation is the obligately anaerobic bacterium of the human GI tract, *Clostridium scindens*. Microbes require macronutrients, micronutrients, and various growth factors like vitamins, amino acids and other organic compounds for their growth. The functions of various micronutrients and vitamins required by bacteria are listed in Table 5.

Table 5. Growth factors: Vitamins and their Functions [45]

Vitamin	Function
p-aminobenzoic acid	Precursor of folic acid
Folic acid	One carbon metabolism; methyl group transfer
Biotin	Fatty acid synthesis; β-decarboxylations; some CO_2 fixation reactions
Cobalamin	Reduction of and transfer of single carbon fragments; synthesis of deoxyribose
Lipoic acid	Transfer of acyl groups in decarboxylation of pyruvate and α-ketoglutarate
Nicotinic acid	Precursor of NAD^+
Pantothenic acid	Precursor of coenzyme A; activation of acetyl and acyl derivatives
Riboflavin	Precursor of FMN; FAD in flavoproteins involved in electron transport
Thiamine	α- decarboxylations; transketolase
Vitamin B_6	Amino acid and keto acid transformations
Vitamin K group; quinines	Electron transport; synthesis of sphingolipids

It was important to see if *C. scindens* was placed closer to other bile acid dehydroxylating bacteria in the phylogenetic tree or not. Through 16S rRNA and *bai* gene hybridization pattern, *C. scindens* was seen to be closely related to *C. hylemonae* and was placed in the phylogenetic tree as shown in Figure 27 [41]. The genes coding for 16s rRNA are the least variable nucleic acids in all cells. Thus it can be used to find out relatedness among distant organisms. This technique is used by scientists to classify different bacteria in their respective taxonomy and phylogeny. It also helps them to estimate the rates of species divergence among bacteria [35].

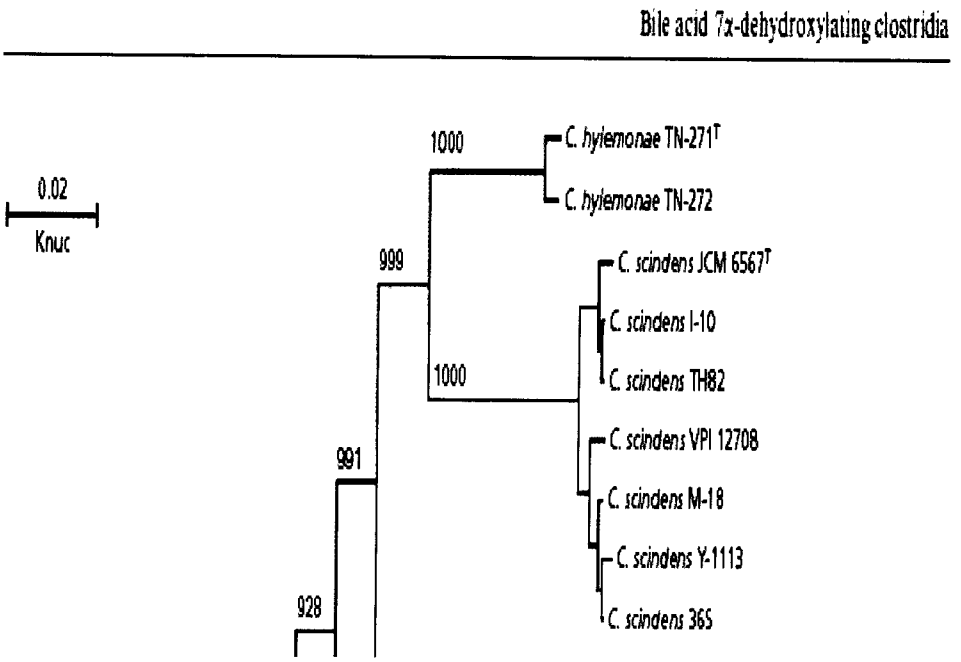


Fig. 27. Phylogenetic relationships within the *Clostridium* cluster XIV and closely related species. The tree was created by using the neighbor-joining method and K_{nuc} values. The numbers on the tree indicate bootstrap values greater than 50% Bar, 0.02 K_{nuc} [38].

It is known that primary bile acids are converted to secondary bile acids by intestinal bacteria by 7α-dehydroxylation. Previous works showed dehydroxylation of primary bile acids under a nutrient rich medium [18, 32, and 73]. To see if under defined medium conditions *C. scindens* VPI 12708 was able to dehydroxylate the primary bile acid cholic acid to the secondary bile acid deoxycholic acid or not, *C. scindens* was grown with sodium cholate in defined medium. To confirm the dehydroxylation of primary bile acids, thin layer chromatography (TLC) was performed under defined culture conditions. And it was observed that even in defined conditions, the bacterium dehydroxylated cholate and converted it to deoxycholate (Fig. 9). Earlier works done by Eneroth in 1963 [18], Hylemon *et al* [32], Stellwag [73], White and Lipsky [82] also show products of dehydroxylation separated by TLC under a nutrient rich condition; however, this is the first time where dehydroxylation by *C. scindens* was observed under defined culture conditions. When the bacterium is grown in a highly defined medium, it has very limited resources, which puts stress on the general anabolic reactions that require input of energy and material. Thus, our results were the first reported instance of *C. scindens* carrying out the energy-consuming process of dehydroxylating primary bile acids under anabolic stress conditions. From this, we can also conclude that the genes that are required for dehydroxylation are still expressed under anabolic stress.

Two unknown products were detected by TLC when *C. scindens* was grown in presence of cholate under defined culture conditions (Fig.16). These unknown products were absent when *C. scindens* was grown under nutrient-rich BHI culture conditions with cholate. Whether these unidentified metabolites were formed during the metabolism of cholate or deoxycholate is presently unknown. However, studies by Heftman, *et al* have observed metabolites/by-products other than the products of dehydroxylation of bile acids [29]. Some of the following metabolites/by-products that was observed by them under defined media conditions is as follows: 3 -β hydroxy -5β cholanoic acid, 3-α hydroxy- 12-keto-5β-cholanoic acid [29]. 3β, 12α –

dihydroxy - 5β cholanic acid, 3β hydroxy – 12 – keto 5β cholanic acid was confirmed by Danielsson, Eneroth *et al* [10]. Norman and Palmer confirmed the presence 3 keto - 5β cholanoic acid [57]. 3α, 12α- dihydroxy- 7 keto - 5β cholanic acid and 3α, 7β, 12α trihydroxy 5β cholanoic acid was confirmed by Hamilton [27]. Eneroth et al confirmed the presence of 3,12 – diketo, 3-keto - 12α – hydroxy, 3α, 12β dihydroxy, 3β, 12β dihydroxy, 3α keto- 7α-hydroxy, 3α hydroxy – 7 – keto, 3β, 7α dihydroxy, 3α, 7α dihydroxy, 3α, 7β dihydroxy [19]. So those two unknown products could be one of the above mentioned products. Also Hylemon showed in his TLC analysis that some of the intermediates separated via TLC were 3-oxo-Δ^4CA, 3-oxo-CA, 7-oxo-CA, 3-oxo-Δ^4 DCA, 3-oxo-$\Delta^{4,6}$-CA, 3-oxo-DCA, and allo-3-oxo-DCA [32]. On comparing the TLC plate from Hylemon to Fig.16, it may be assumed that the two unknowns maybe 3-oxo-CA and 7-oxo-CA, also with the potential of one of them being 3-oxo-Δ^4-CA.

Not much is known about the physiology of *C. scindens* other that the fact that it dehydroxylates cholic acid and converts it to deoxycholic acid. As a result it was necessary to study the physiology of this organism and figure out its nutritional requirements. From previous studies it was found that the bile acid-dehydroxylating bacterium *C. bifermentans* required biotin, nicotinic acid, pantothenic acid and pyridoxal for its growth [68]. *C. aceticum,* another clostridium species but not a bile acid dehydroxylating organism, requires biotin, pantothenic acid and pyridoxamine [37].

As no previous work has been done to resolve the vitamin requirements of *C. scindens*, one of the objectives of the study was focused on resolving its vitamin requirements. Riboflavin and pantothenate were found to be essential for the growth of *C. scindens*. When these two vitamins were absent very little growth was observed on the first passage, possibly due to carry-over of trace levels of this vitamin from the inoculum, and no growth was detected in the subsequent second passage.

Pantothenate typically acts as the precursor of coenzyme A [45]. It is also required for the activation of acetyl and acyl derivatives [45]. Thus this vitamin is important for enzymatic reactions involving coA, including the Krebs cycle. The biosynthesis pathway of pantothenate in *Escherichia coli* K-12 MG1655 is shown in Figure 28 [15]. The various genes encoding the different essential enzymes are *panB, panE, panC,* and *panD.* When the sequence of each of the proteins of these genes was aligned against the whole genome sequence of *C. scindens* ATCC 35704 using BLAST, it was observed that the genes *panB, panC, panE* and *panD* are probably absent in *C. scindens* as the E-values of these genes are higher than the cut-off value (see results).

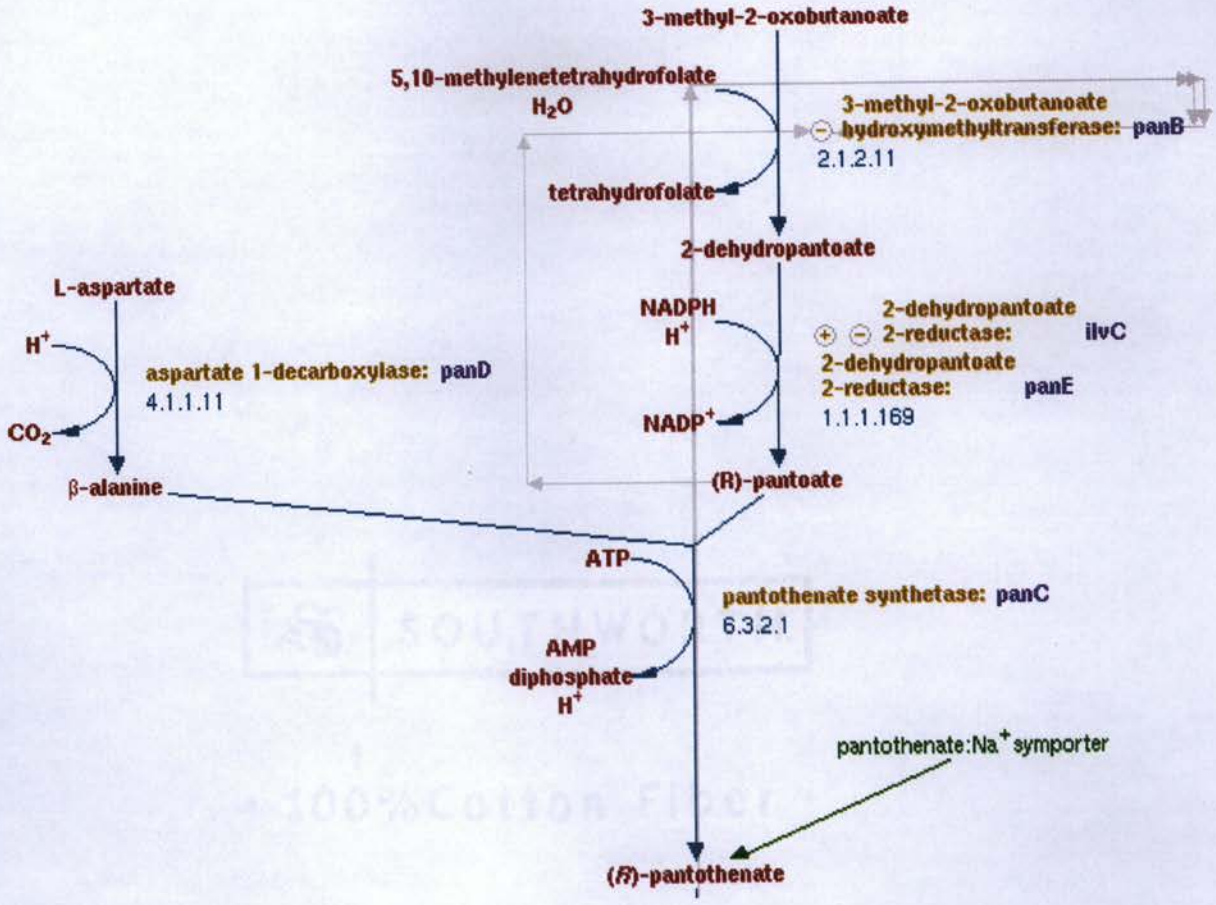


Fig. 28. Biosynthesis of pantothenate in *E. coli* K-12 strain MG1655[15].

The function of each of the genes involved in pantothenate synthesis is discussed in detail below.

***pan*B:** In pantothenate biosynthesis, the first committed step is catalyzed by 3-methyl-2-oxobutanoate hydroxymethlytransferase [36]. Ketopantoate is formed from condensation of formaldehyde with α-ketoisovarlerate in this step [36]. The enzyme was purified by Teller et al from wild type *E. coli* [78]. It has been seen by Cronan that *pan*B mutants are pantothenate auxotrophs [9]. Hence this may suggest the requirement of pantothenate by *C. scindens* for its growth. From the blast analysis it was seen that there was no significant match for *pan*B gene in *C. scindens* with *E. coli* (E-value – 0.82).

***pan*E:** Pantoate is formed from the reduction of ketopantoate [21]. The enzyme that carries out this NADPH-dependent reduction is 2-dehydropantoate-2-reductase. This enzyme is coded by *pan*E.

***pan*C:** The synthesis of patothenate from pantoate and β–alanine is catalyzed by pantothenate synthetase and requires hydrolysis of ATP [25]. This enzyme is coded by *pan*C [25]. *pan*C mutants were found to be auxotrophic for pantothenate [56].

Both *pan*E and *pan*C had E-values 1.0 and 0.060 respectively much higher than the significant match cut-off suggesting no significant match for these genes were found in *C. scindens* VPI 12708.

Thus it can be concluded that all the main genes that code for the enzymes catalysing the pantothenate biosynthesis in *E. coli* are absent in *C. scindens* VPI 12708. As these genes are absent, *C. scindens* VPI 12708 cannot synthesize pantothenate on its own and does not grow in a medium lacking pantothenate.

Another vitamin which is essential for growth of *C. scindens* VPI 12708 is riboflavin. Riboflavin acts as a precursor of FMN and FAD, which are flavoproteins involved in electron transport [45]. Figure 29 outlines the biosynthetic pathway of riboflavin [15].

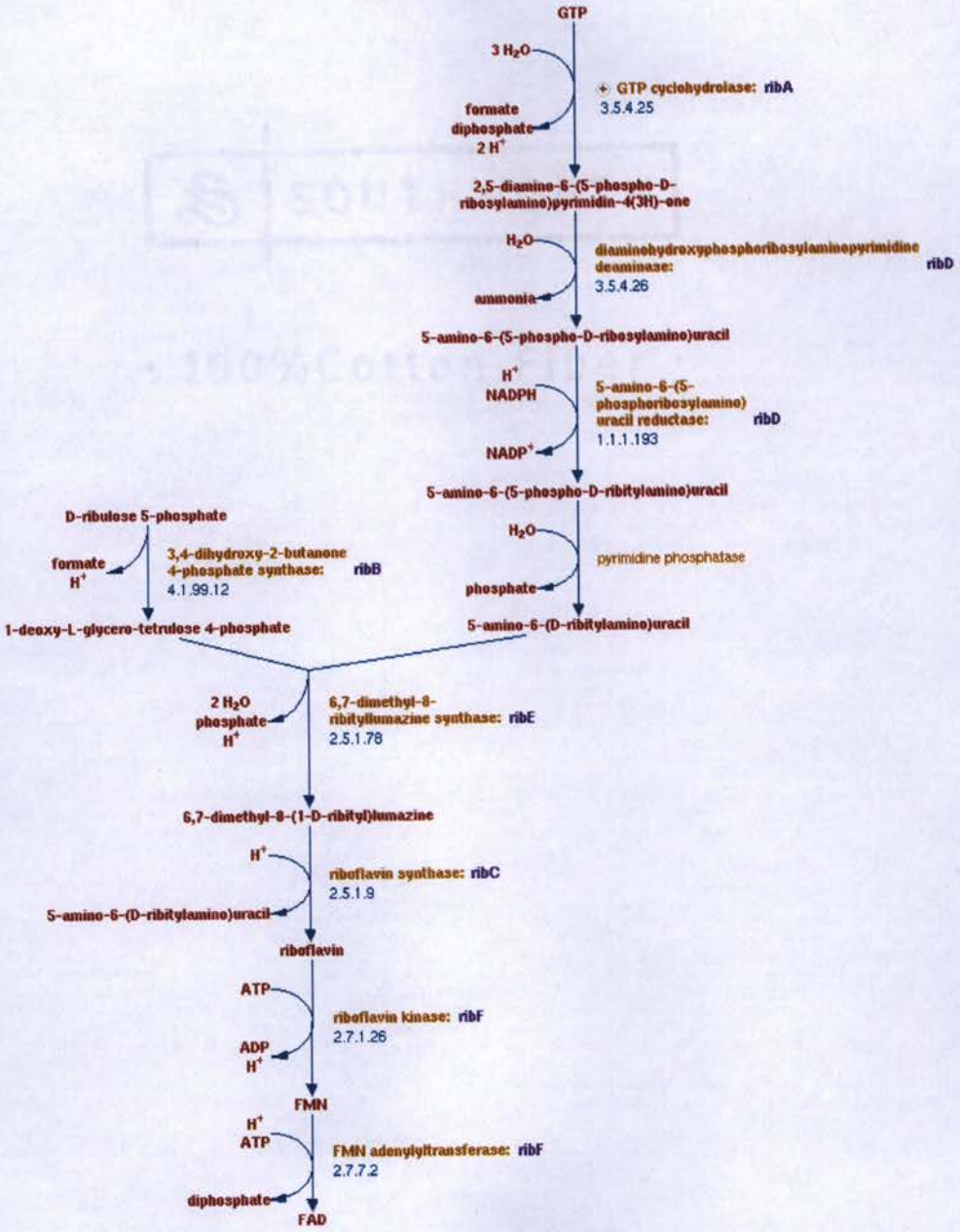


Fig. 29. Biosynthetic pathway of riboflavin in *E. coli* K-12 strain MG1655. [15]

ribD, ribA, ribB, ribE, ribC, ribF are the various genes encoding the different enzymes in the riboflavin biosynthetic pathway [15]. When the sequence of each of these genes from the riboflavin pathway was aligned against the whole genome sequence of *C. scindens* ATCC 35704, it was observed that the genes *ribA*, *ribB, ribC, ribD* and *ribE* are probably absent in *C. scindens* as the E-values of these genes are higher than the cut-off value (See results). The function of each of the genes is discussed in detail as below.

***rib*A:** In *E. coli* K-12 substrain MG1655, *ribA* gene codes for the enzyme GTP cyclohydrolase II [63]. This enzyme was first isolated from *E. coli* cell extracts by Forrest Foor and Gene M. Brown [20]. The release of the eighth carbon from the imidazole ring of GTP as formate and the removal of pyrophosphate from the triphosphoribosyl side chain is catalyzed by GTP cyclohydrolase II [3]. Magnesium ions are required for the enzyme's activity [3]. It is the first committed step in riboflavin biosynthesis [62]. Since the first step of the overall reaction is the hydrolytic cleavage of the imidazole ring of GTP, the enzyme was named GTP cyclohydrolase [63]. By Baba *et al*, *rib*A is considered an essential gene [2]. As no significant match was found in *C. scindens* for *rib*A (E value = 4.3) the absence of this gene maybe assumed and this shows the bacteria's inability to synthesize its own riboflavin and requirement of riboflavin in the growth media.

***rib*B:** In *E. coli*, the enzyme 3, 4 dihydroxy-2 butanone-4-phosphate synthase is coded by *rib*B gene [62]. In the biosynthesis of riboflavin, the enzyme catalyzes the formation of a four carbon compound called 3, 4 dihydroxy-2butanone-4-phosphate which condenses with 5-amino-6-ribitylamino-2, 4—pyrimidinedione to give 6, 7-dimethyl-8-ribityllumazine which is a direct precursor of riboflavin [62]. This gene is also considered as an essential gene [2]. The E-value for this gene in *C. scindens* was 1.1 which is not considered very significant and hence the assumption of the absence of this gene.

***rib*D:** *rib*D encodes for a bifunctional enzyme in *E. coli* which catalyzes the deamination of 2, 5-diamino-6-ribosylamino-4-pyrimidinone 5'-phosphate to 5 amino-6-ribosylamino-2, 4-pyrimidinedione 5'-phosphate and subsequent reduction of the ribosyl side chain [64].

***rib*E:** In the riboflavin biosynthesis pathway, lumazine synthase catalyzes the penultimate step and is coded by *rib*E gene [54].

***rib*C:** The final step in the riboflavin biosynthesis pathway is catalyzed by riboflavin synthase [14]. Riboflavin is produced from 6, 7-dimethyl-8-ribityllumazine by an unusual dismutation catalyzed by this enzyme [14]. *rib*C gene codes for riboflavin synthase in *E. coli* [60].

*rib*D, *rib*E and *rib*C are also essential genes [2]. The E-values for these genes in *C. scindens* were not very significant ($1e^{-06}$, 4.5 and 0.43 respectively). Hence the absence of these genes in *C. scindens* maybe assumed. As a result, the bacteria cannot synthesis its own vitamin and does not survive if absent from the growth medium. It can be concluded that riboflavin is an essential vitamin.

Though riboflavin and pantothenate showed clear requirement for the growth of *C. scindens* VPI 12708, there was a conflict among biotin and pyridoxal requirement for the bacteria. During initial experiments both biotin and pyridoxal were required but on follow up experiments to verify these findings results indicated these two vitamins were not required for the growth of *C. scindens* VPI 12708. There is some minor evidence to suggest that the biosynthetic pathways of biotin and pyridoxamine are interconnected [55]. This may account for the differences in the results that were observed using the leave one out technique, even though biotin was left out of the solution, pyridoxal was present to fulfil the growth requirements of *C. scindens* and vice versa. This is mere speculation at this point and must be substantiated in the future with additional studies.

However, various studies performed by different scientists on *E. coli* to figure to the biotin requirement of E. coli K-12 strain MG1655 have found that *E. coli* can survive without the presence of biotin under certain circumstances as described: Eisenberg and Star noted that a compound which can replace biotin requirement for growth in yeast cells is formed when pimelyl-CoA combines with alanine in the presence of pyridoxal phosphate [17]. In several microorganisms, the biotin requirement has been replaced by pimelic acid, desthiobiotin, oxybiotin, aspartic acid, oleoic acid and sometimes also by certain intermediates of Tricarboxylic acid cycle [7]. In certain *E. coli bioD* mutants, it was observed that they were able to grow on dethiobiotin and did not require biotin for growth [13]. Maybe the *C. scindens* VPI 12708 can also survive without biotin as the other vitamins and amino acids might replace the requirement for biotin. In studies done by Lam H.,M. and Winkler M.,E., they observed that *E. coli* having *Pdx*H suppressor mutations grow without pyridoxal suggesting "the presence of an alternate pathway or form of oxidase for PN oxidation in *E. coli*" [40]. In a study by Man T. K., by creating suppressor mutations, growth of Δ*pdx*H::Ω mutant without pyridoxal was observed [46]. *Pdx*H is the gene that encodes the enzyme pyridoxine-5'-phosphate oxidase that is required in the final step of the pyridoxal pathway where pyridoxine-5'-phosphate is converted to pyridoxal-5'-phosphate [15]. Maybe due to the presence of an alternative pathway for oxidase or an alternate form of oxidase, the function of *pdx*J and *pdx*H is by-passed and the organism can grow in the medium even though lacking the genes and the medium lacking pyridoxal. Also in the same study by Man T.,K., it was found that *pdx*J mutants could grow without pyridoxal and also that the mutation could bypass the requirement of *pdx*H gene [46]. They also assume that due to the mutation in the *pdx*J gene, the *pdx*J protein acts as an activator of a "cryptic" biosynthetic pathway for pyridoxal biosynthesis [46].

After the vitamin requirements were resolved, the amino acid requirements were determined. In the past it was seen that for the germination of spores of *C. sordellii*, alanine,

phenylalanine and arginine were required [61]. Nothing is mentioned if amino acids are important for the growth of *C. sordellii* or not. For *C. bifermentans*, though no amino acid was required for its growth, it was observed that alanine, phenylalanine, arginine was required for the germination of spores [80]. For *C. scindens* we found that only one amino acid tryptophan was required for its growth. Thus like for many organisms it looks like it is an essential amino acid for *C. scindens* as it cannot synthesize tryptophan on its own. The pathway for tryptophan is described in detail as follows and the pathway is as shown in Figure 30.

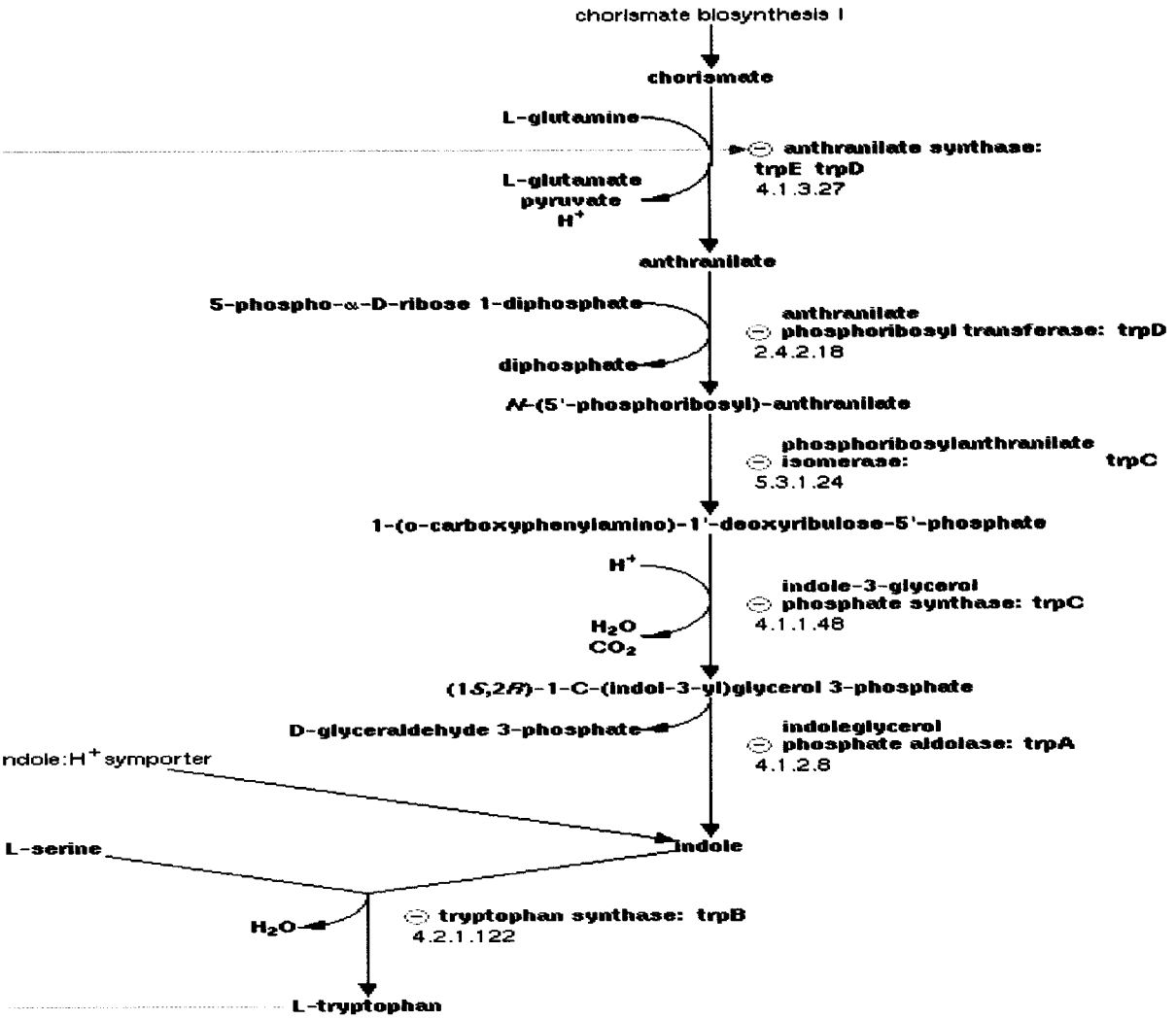


Fig. 30. Tryptophan Biosynthesis pathway in *E. coli* K-12 strain MG1655[15].

Chorismate, the principal precursor of various amino acids like tyrosine, phenylalanine, and other essential compounds like tetrahydrofolate, ubiquinone-8, menaquinone-8, and enterbactin produces tryptophan in *E. coli* [15]. The biosynthesis of tryptophan is a complicated and energetically expensive process as it requires D-erythrose-4-phosphate, phosphoenolpyruvate and ATP for biosynthesis of chorismate first and then requires glutamine, serine, and phosphoribosyl pyrophosphate for tryptophan biosynthesis from chorismate [15].

The five enzymes that catalyze the conversion of chorismate into tryptophan are encoded by five genes namely *trp*E, *trp*D, *trp*C, *trp*B and *trp*A [15]. These five genes together form a transcriptional unit called the *trp* operon [15].

***Trp*E** (sometimes *Trp*DE) codes for the enzyme anthranilate synthase which catalyses the first step in the biosynthesis of tryptophan [58]. Anthranilate and glutamine are formed from chorismate after anthranilate synthase catalyzes the glutamine amidotransferase reaction which adds an amine group from glutamine to chorismate [4, 34].

***Trp*D:** In the second step of tryptophan biosynthesis, N-5'-phosphoribosyl-anthranilate is formed from anthranilate which is catalyzed by anthranilate phosphoribosyl transferase [41]. This enzyme is encoded by *trp*D [41].

***Trp*C:** The third and fourth steps in the biosynthesis of tryptophan are carried out by a bifunctional enzyme- phosphoribosylanthranilate isomerase/indole–3–glycerol phosphate synthase which is encoded by *trp*C gene [15, 70]. In the third step of the pathway the phosphoribosylanthranilate isomerase part of the enzyme catalyzes the reaction:

Phosphoribosylanthranilic acid ⟶ 1-(o–carboxylphenylamino)-1-deoxyribulose 5-phosphate (CDRP) [69]

The indole–3–glycerol phosphate synthase part of the enzyme catalyzes the following reaction:

CDRP ⟶ Indole – 3- glycerol phosphate [69]

***Trp*A:** The fifth step in tryptophan biosynthesis where indole is formed from indole – 3 – glycerol phosphate by the aldol cleavage with the elimination of D- glyceraldehyde 3– phosphate is catalyzed by the tryptophan synthase multienzyme complex consisting of $\alpha_2\beta_2$ subunit [15, 69]. TrpA codes for the α subunit [83].

***Trp*B:** In the final step of tryptophan synthesis, indole combines with L-serine releasing water to produce tryptophan [42]. This reaction is catalyzed by the β_2 subunit of the multienzyme - tryptophan synthase [42]. The β subunit is coded by *trp*B [83].

Based on the BLAST results, the E-values for *trp*D, *trp*E, *trp*C, *trp*A and *trp*B were $5e^{-09}$, 0.96, 0.87, 0.28 and $4e^{-05}$ respectively which are higher than the cut-off for considering them to be a significant match. Our BLAST results of protein sequences showed that none of these 5 genes are present in *C. scindens* VPI 12708. This would explain the tryptophan auxotrophic phenotype of *C. scindens* VPI 12708 in defined growth medium.

Although *C. scindens* is a model for studying the dehydroxylation of primary bile acids due to its high activity in the pathway, very little was known about the nutritional physiology of *C. scindens*. This study elucidated a defined growth medium recipe that requires the presence of two vitamins, riboflavin and pantothenate, as well as one amino acid, tryptophan, to support anaerobic growth of *C. scindens*. Furthermore, *in vivo* dehydroxylation of primary bile acids were observed in *C. scindens* when it was cultivated under anabolic stress conditions, suggesting a central role for this energy-consuming process in this organisms survival. These new information will help future research on the topics of metabolic flux and regulation of bile acid dehydroxylation.

Conclusions

- From TLC analysis, it was seen that *C. scindens* dehydroxylated the primary bile acid cholic acid to secondary bile acid deoxycholic acid when grown in BHI and DM with 1 mM cholate.
- BHI broth supported growth of *C. scindens* better than other growth media tested.
- When the complete vitamin solution and complete amino acid solution was removed from DM, growth of *C. scindens* was not supported.
- When DM was supplemented with both vitamin and amino acid solutions (see DM + CVS + CAAS), both served as effective source of growth factors and supported the growth of this gut bacterium.
- When DM was supplemented with the complete amino acid solution or the complete vitamin solution alone, *C. scindens* showed no growth.
- Upon removing glucose from DM (see DM–G + CVS + CAAS), growth of *C. scindens* was not supported, indicating that this bacterium required glucose as an energy source.
- When the individual vitamins were removed from the complete vitamin solution and supplemented with DM, it was seen that riboflavin, pantothenate, pyridoxal and biotin were required by *C. scindens* for growth.
- On confirmation experiments designed to resolve vitamin requirements, produced conflicting results and as a result the vitamin requirements remain unresolved.
- When the different amino acid groups were removed from the complete amino acid solution and supplemented with DM, it was seen that the amino acids from the aromatic group were the ones required by *C. scindens* for growth.

- By supplementing the DM with different combinations of amino acids from the aromatic group, it was seen that tryptophan was required by *C. scindens* for growth.

- Based on BLAST results, it was seen that the proteins of all the *pan* genes, *trp* genes and *rib* genes except *ribF* are assumed to be absent in *C. scindens* VPI 12708.

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